BLOOD PRESSURE RESPONSES RELATED TO TYPES OF ANIMAL PREPARATION

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During a long series of studies on the aetiology of experimental renal hypertension, using the clamp method for inducing this state (Goldblatt, Lynch, Hanzal and Summerville, 1934) as modified by Wilson and Byrom (1941) for rats, it has recently appeared that two factors have not been completely resolved in the interpretation of results obtained. One is the choice of the animal preparation used for assay and the second is the method by which pressor responses are finally measured.

A reasonably intact animal preparation is considered to be prerequisite for the testing of pressor substances. One might include in this category bilaterally nephrectomized rats if they are used within a period of eighteen hours after operation. This type of animal preparation undoubtedly shows increased sensitivity to some pressor agents, notably to renin (Braun-Menendez, Leloir, Fasciolo, Munoz and Taquini, 1946). Normal, unilaterally and bilaterally nephrectomized animals of different species have been used extensively for blood pressure assays.

Intimately related to these facets in the determination of blood pressure is the suspected participation of the adrenal glands in a primary or subsidiary rôle. This applies both in the maintenance of normal blood pressure and in the implication that the adrenal glands are involved in the pathogenesis of experimental renal hypertension (Knowlton, Stoerk, Seegal and Loeb, 1946). Experimental findings are somewhat analogous to observations in patients with Addison's disease. In two early reported series (Ferrebee, Ragan, Atchley and Loeb, 1939; Soffer, Engel and Oppenheimer, 1940), patients were treated by each group of workers respectively with desoxycorticosterone in oily solution and DCA pellets. Blood pressures of hypertensive level occurred in several patients of each series.

The advent of some standardized adrenal cortical hormones has widened the scope of possible animal preparations besides indicating other means of

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approach to the central problems of maintenance of blood pressure and hypertensive states. As a consequence it was decided to use for assay animals which had been pretreated with adrenal cortical hormones, both synthetic and natural, and subjected to different arrangements of operative procedure.

It appears that the rise in blood pressure and the maintenance of this rise is usually recorded as the height of the increase in blood pressure and the time during which it is sustained. It was felt that a better correlation and more information might be gained if the pressor-time response was measured by planimetric methods.

The experiments reported were based on these two assumptions regarding adrenal cortical influence and the planimetric measurement of responses.

Methods.

Twelve subgroups with three rats in each were used. The animals were male Wistar albino descendants approximately 200 gm. in weight. In the adrenal cortical hormone subgroups, desoxycorticosterone in sesame oil was given intramuscularly in two daily doses of 1 mg. each, commencing 48 hours before test injections; cortisone in saline suspension was injected subcutaneously at a 5 mg. dosage level 48 and 24 hours before testing. The operative procedures tabulated in the results were carried out eighteen hours before final testing and all animals, including controls, were given 1 p.c. saline to drink.

The two pressor substances chosen to test on these differently prepared rats were renin (Haas, Lanfrom and Goldblatt, 1954) and adrenaline solution which was freshly made up from powder. The respective doses by intravenous injection into the exposed jugular vein were: renin 0·5 ml. and adrenaline 0·0001 mg. The test animals were anaesthetized with ether and the carotid artery cannulated after heparin administration. The blood pressure effects after injection were recorded directly through a mercury manometer with kymograph tracing.

Results were then subject to planimetric measurement commencing from the time of injection to an arbitrarily fixed end-response and returning to the original point. In the case of adrenaline, four minutes were chosen and this meant, in some instances, that the depressor phase of adrenaline had been entered into, so the pointer was taken obliquely back to the point of injection. Ten minutes was the period selected for renin and in order to complete the pressor time area survey, the planimetric pointer was taken vertically down to the injection level and then back horizontally to this initial mark.

RESULTS.

The figures presented in the Table are those which were obtained by the planimetric methods described above, the area being measured in square centimetres. All rats were in good condition during the time of the experiment and the subsequent period of anaesthesia for direct blood pressure determinations. Figs. 1 to 4, which are annotated, are representative of the subgroups they illustrate. A second injection of renin which was given to every rat tested showed the development of tachyphylaxis of varying degree. The form of the curves of blood pressure response was relatively constant within each subgroup.

TABLE 1.*

Group (Type of operation)	Subgroup (+Preassay injection)	Test substance	
		Adrenaline (mean)	Renin (mean)
1. Normal	A. + Nil	1·9	7·5
	B. + Cortisone	1·2	12·3
	C. + DCA	1·8	10·0
2. Bilaterally adrenalectomised	D. + Nil	1·8	9·3
	E. + Cortisone	1·6	11·0
	F. + DCA	1·7	8·7
3. Bilaterally nephrectomised	G. + Nil	1·1	13·4
	H. + Cortisone	1·0	9·8
	K. + DCA	2·5	10·3
4. Bilaterally nephrectomised with bilateral adrenalectomy	L. + Nil	4·9	14·6
	M. + Cortisone	1·3	13·0
	N. + DCA	1·9	18·3

* The figures show the area in square centimetres of time-pressor response.

DISCUSSION.

One criticism which might be advanced is that the number of animals in each group was small, especially for assay purposes. Secondly, no serum electrolyte nor protein studies were carried out. However, as the types of animal preparation generally used for testing pressor substances have been controversial in relationship to species and pre-injection treatment, the results demonstrate several features of interest without considering these other factors at the moment. The pressor effects of adrenaline and renin have long been known to be different in time response and the nature of the curves obtained, and were almost automatic selections for this experiment. It is probably most convenient to analyze the blood pressure responses to adrenaline and renin separately and then point out the disparities between the results with each and both substances.

As far as adrenaline was concerned, the greatest response was given by subgroup L, where there was marked prolongation of reaction (Fig. 3). A striking feature was the uniformity of response in all subgroups of those animals pretreated with DCA. By way of contrast, the untreated and cortisone-treated subgroups behaved more erratically, mostly by lesser responses and more variation, although there were several exceptions to this.

Renin also produced some discordant results. Normal rats showed the least B.P. response, but the level was much higher with cortisone and DCA pretreatment (subgroups B and C). The nephrectomized-with-adrenalectomy subgroups produced greater responses than any other subgroups. Interestingly, subgroup N, in the renin series, gave the most marked blood pressure time response.

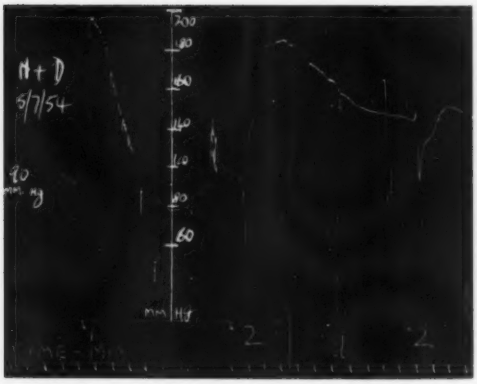

Fig. 1. Subgroup C. Sharp intermediate rise is due to endogenous adrenaline secretion from light anaesthetic.

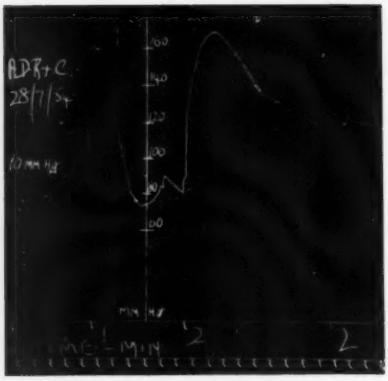

Fig. 2. Subgroup E. No response to second injection of renin.

(1 = adrenaline. 2 = renin)

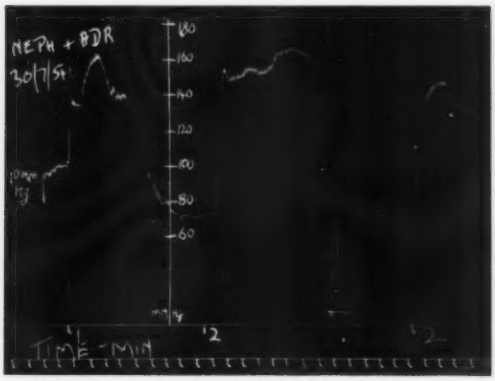

Fig. 3. Subgroup N. Well marked response to a second injection of renin.

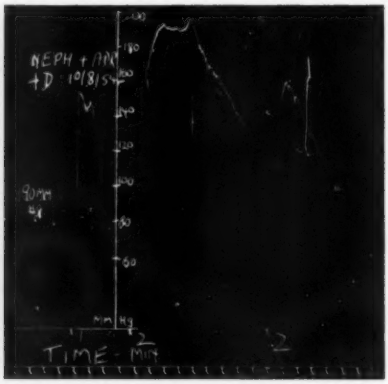

Fig. 4. Subgroup L. Considerable widening of the adrenaline curve (1) and sustained response to renin (2).

(1 = adrenaline. 2 = renin)

Adrenal insufficiency diminishes the pressor reaction to renin and the administration of cortical extracts restores responsiveness (Remington, Collings, Hays, Parkins and Swingle, 1941). Although the rats of group 2 could scarcely be graded as having had adrenal insufficiency, it is interesting that this group

provided the lowest readings to renin and that cortisone was the more successful adrenal cortical hormone in restoring near normal B.P.-time reading. Adrenaline response, on the other hand, was maintained by both DCA and cortisone. In the normal animals, adrenaline reaction was maximal in subgroup C and renin reaction in subgroup A the least of all recorded. It is difficult to postulate an hypothesis to explain the low renin response in subgroup A with intact adrenals and kidneys if it is considered that competitive, activating or inhibitory actions may exist between the different cortical hormones. When the possible rôle of cortical hormones in the destruction of renin and hypertensin or the augmentation of the action of hypertensinase (Fasciolo, Leloir, Munoz and Braun-Menendez, 1940) is added to these problems, the difficulties are multiplied many times.

Tigerstedt and Bergman (1898) demonstrated that animals were more sensitive to renin when they were bilaterally nephrectomized and this observation has been confirmed many times since then, and group 3 was in accord with these numerous observations. More striking were the results in group 4 where a high reading in each subgroup was recorded, especially in subgroup N. There was a scatter in the results of each subgroup, probably due to the extensive operative procedures combined with subsequent anaesthesia. Some such hypersensitivity was shown with adrenaline where there was prolongation of the action of adrenaline in subgroup L (Fig. 3).

The planimetric measurements were simple to carry out, and, if taken to the end point of pressor response, might well have yielded more information.

SUMMARY.

The pressor responses of rats: normal, pretreated with DCA and cortisone are presented. Each subgroup had either no injection, cortisone or DCA. Groups included normal, adrenalectomized, nephrectomized and nephrectomized+adrenalectomized rats. Discrepancies between the different groups are discussed. The preinjection of DCA into assay animals proved to be a more stabilizing influence to subsequent adrenaline injection than cortisone pretreatment. When renin was the test substance, cortisone-treated animals behaved more consistently than DCA-treated rats; and this was especially notable in the bilaterally nephrectomized+adrenalectomized subgroup which gave a most pronounced response to renin after pretreatment with DCA. Planimetric measurements were used to determine the pressor-time response.

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REFERENCES.

Braun-Menendez, E., Fasciolo, J. C., Leloir, L. F., Munoz, J. M. and Taquini, A. C. (1946): Renal Hypertension, p. 120. Charles C. Thomas, Springfield, Illinois.

Fasciolo, J. C., Leloir, L. F., Munoz, J. M. and Braun-Menendez, E. (1940): Rev. Soc. Argent. de biol., 16, p. 643.

Ferrebee, J. W., Ragan, C., Atchley, D. W. and Loeb, R. F. (1939): J. Amer. med. Assoc., 113, p. 1725.

Goldblatt, H., Lynch, J., Hanzal, R. F. and Summerville, W. W. (1934): J. exp. Med., 59, p. 347.

Haas, E., Lanfrom, H. and Goldblatt, H. (1954): Arch. Biochem. Biophysics, 48, p. 256.

Knowlton, A. I., Stoerk, H. C., Seegal, B. C. and Loeb, E. N. (1946): Endocrinol., 38, p. 315.

Remington, J. W., Collings, W. D., Hays, H. W., Parkins, W. M. and Swingle, W. W. (1941): Amer. J. Physiol., 132, p. 622.

Soffer, L. J., Engel, F. L. and Oppenheimer, B. S. (1940): J. Amer. med. Assoc., 115, p. 1860.

Tigerstedt, R. and Bergman, P. G. (1898): Arch. Physiol., 8, p. 223.

Wilson, C. and Byrom, F. B. (1941): Quart. J. Med., 10, p. 65.

A REMOTE-CONTROLLED MICRO INJECTOR

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Administration of drugs is a basic step of much physiological research. However, the emotional stress to the animal which occurs as a result of injection when this is required may initiate a series of bodily changes which interfere with the experiment. This is particularly so in those problems where alteration in the activity of the sympathetic adrenal system can modify the response of the animal. We were faced with the above problem and therefore developed a simple micro injector which is capable of being operated by remote control. The whole injector can be buried in an animal so that a single injection of 0·02 ml. to 0·2 ml. can be given into a known site when desired without disturbing the animal.

EXPERIMENTAL.

Principle of micro injector.

A compressed spring is held away from a rubber-capped glass tube by means of a metal disc made from a low melting point alloy. At the time of the injection the animal is placed near the coils of a high frequency induction heater. When the coils are activated the metal alloy disc melts and the spring bears down on the rubber cap and ejects the required volume of the drug from the glass tube.

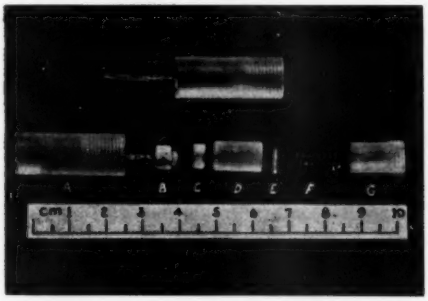

Fig. 1. Component parts and assembled micro injector. For details see text.

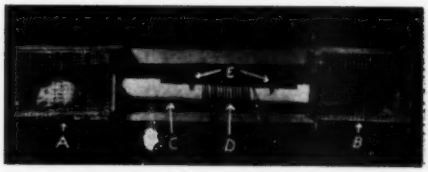

Fig. 2. Assembly used to operate micro injector when animals move through the fibreboard tube. The high frequency induction heater is not shown.

[1]Working with a full-time grant from the National Health and Medical Research Council.

Details of construction.

A photograph of the assembled apparatus and component parts is presented in Fig. 1. This is suitable for use in the rat. The injector consists of:—

A. A perspex tube of internal diameter 7–8 mm. with an internal thread at one end and a 2 mm. hole in the centre of the other.

B. A drug container which consists of a glass tube with tube grip and attached polyvinyl chloride (p.v.c.) tubing at one end and sealed with a rubber cap at the other. We have found the rubber tops of fountain pen tubes to be most satisfactory for this purpose. The length and diameter of the cap controls the volume injected.

C. A perspex plunger which is a clearance fit inside D.

D. A perspex sleeve fitting tightly into tube A.

E. A metal disc 1 mm. thick with a diameter the same as the outside diameter of the sleeve D. This is made from Wood's metal which has a melting point of 65° C.

F. A steel spiral spring made from 28 gauge (British Imperial) wire with 4 cm. free length and 15 turns.

G. A perspex cap with external thread to match that on tube A. A clearance hole for the spring F is drilled to within 3 mm. of the end of the cap. The length of the hole is such that it will just accommodate the spring when compressed.

Assembly. The drug container B is filled by alternate compression and suction. During this procedure the effectiveness of the rubber top glass seal is tested. Air bubbles are removed from the drug container during filling by allowing the container to hang vertically whilst the pressure is slowly released. The p.v.c. tube attached to the tube grip is sealed with a wire plug. The container and contents are sterilized by heating. Alternatively, for drugs which are unstable to heat, the container can first be sterilized and then be filled with the drug. The container B and plunger C are placed in the sleeve B, which is inserted to its full extent into the tube A. The metal disc is next put into this tube so that it will rest against the sleeve D. This is followed by the spring F and cap G, the latter being screwed tightly into position. The spiral spring is thus held compressed between the cap and disc, the latter preventing the spring from pressing on the plunger. The whole apparatus is enclosed in a latex bag so that only the tube grip and attached p.v.c. tubing are exposed. The outside of the assembled injector is sterilized by immersion in 1:1,000 zephiran for 15 minutes and is then ready for insertion into the animal. The complete apparatus weighs 7 gm.

Operative technique.

The needle, connecting tube and injector are implanted as described for the continuous micro injector (Rose and Nelson, 1955). The only difference is that at the second stage the connecting tube and needle are filled with the drug just prior to the connection of the injector. Pre- and post-operative care are similar to that described elsewhere. Animals should be allowed at least 14 days to recover after the second stage operation before continuation of the experiment. Before and during the recovery period the animals are trained to move, when permitted, from cage A to cage B (shown in Fig. 2) *via* the connecting fibreboard tube (C). A number of turns of copper tubing (D) is wound around the central part of this tube and the ends prepared for connection to a high-frequency induction heater. Two perspex plates E may be fitted into

the tube C in order to restrain the animals in this portion of the tube. When the animals have been trained so that they will walk through the tube and submit to the necessary restraint without apparent disturbance they are ready for use.

The training period normally extends over two to three weeks using one training period per day. At the time of the experiment the induction heater is connected and the animals again allowed to walk through the tube. During this procedure the wire spring (F) and alloy disc (E) of the injector are heated so that the latter melts and allows the spring to push the plunger (C) firmly against the rubber cap of the drug container. Thus the drug solution is injected through the needle to the animal. Prior testing has shown that the temperature of the external surface of the injector does not rise appreciably during the procedure. A series of 30 animals have undergone this operation and have, as a result, received a subcutaneous injection of 0·02 ml. of saline. Three hours after the injection the animals were sacrificed and the adrenals removed for the estimation of ascorbic acid and cholesterol. In all cases the values obtained were within normal limits.

SUMMARY.

A simple, remote-controlled micro injector suitable for implantation into animals is described. The animal can thus receive a single injection of 0·02 to 0·2 ml. of drug by remote control and therefore without emotional disturbance.

REFERENCES.

Rose, S. and Nelson, J. F. (1955): Austral. J. exp. Biol., 33, p. 415.

A CONTINUOUS LONG-TERM INJECTOR

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The quantitative measurement of the dose response relationship is an important aspect of physiological and pharmacological research. It is well known that the mode and frequency of drug administration can markedly alter the quantitative effect of a given dose. Certain experiments require that the drug be given as a continuous injection over a long period extending into many weeks. The problems involved in meeting this requirement have not so far been solved and so research workers still inject these drugs once or twice *per diem*. In many such experiments errors in the interpretation of the results will arise as a consequence of the mode of injection. This is particularly so when an attempt is made to estimate the replacement dose of a hormone after the removal of the gland. To approach physiological conditions continuous injection of the hormone is necessary. In other experiments one may wish to deliver the drug continuously for many weeks to one particular site. This will overcome the objections to systemic administration when the site of action of the drug is being studied. Thus, when a drug is given systemically, it—

(i) may not reach the site of action in sufficient concentration;
(ii) may be converted into a form in which its activity is increased or decreased;
(iii) may act first in another organ which, in turn, affects the tissue under particular study.

We were faced with the above problems and therefore developed a simple long-term injector apparatus which is capable of delivering fluid continuously and at a relatively constant rate. The whole device can be buried in an animal which can then lead a reasonably normal life whilst the injection is going on. In conjunction with suitable needles and connecting tubing, drugs, hormones, etc., may be delivered directly and constantly to various organs for many weeks.

EXPERIMENTAL.

Principle of injector.

The motive force of the injector depends on the osmotic pressure developed by a saturated aqueous solution of Congo red against water. This solution is

[1]Working with a full-time grant from the National Health and Medical Research Council.

contained in a partially collapsed rubber compartment and is separated from a second water compartment by a semipermeable cellophane membrane. By osmosis water moves into the Congo red solution and expands the rubber compartment. It is this expansion which provides the mechanical force to eject the drug out of the apparatus.

Details of construction.

Preparation of Congo red. We have used a commercial grade of Congo red which was dialysed against repeated changes of distilled water for eight days. This is necessary because the presence of even small quantities of electrolyte causes an association of the Congo red molecules and so reduces the osmotic pressure (Bayliss, 1909). Congo red has a molecular weight of 697·6 and is soluble in distilled water to about 16 p.c. at 36° C. This would give a theoretical value of 5 atmospheres for the osmotic pressure of a saturated solution at this temperature. We have not been concerned with attaining this theoretical value and in practice only half this value is obtained. This is due to the presence of small quantities of electrolyte which have remained adsorbed on the Congo red. In addition, we used distilled and not conductivity water to make up our solution.

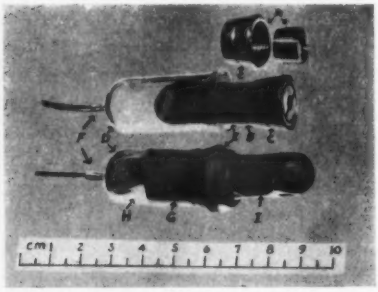

Fig. 1. Component parts and assembled micro injector. For details see text.

Fig. 2. Rat with micro injector implanted subcutaneously.

The long-term injector. Fig. 1 shows the component parts and the assembled apparatus. It consists of three compartments and a clamp to hold the semipermeable cellophane membrane. A simple two-piece screw clamp (A) of monel metal is used to hold a circular disc of cellophane. Monel metal was used because it does not corrode and is more readily machined than steel. It is important to have the two surfaces which clamp the cellophane machined flat to obtain a watertight seal. We have used a good grade of cellophane 0·075 mm. thick. When 16·5 square mm. are exposed the injector delivers fluid at 0·12 ml. per diem. However, any desired rate can be achieved by using a suitable cellophane and an appropriate sized hole in the clamp. The Congo red compartment consists of a latex bag (B) of 3·0 ml. capacity. A stainless steel ball 3 mm. in diameter, 0·5 ml. of distilled water and 0·6 gm. of Congo red are placed in the bag. This makes a saturated solution with 0·52 gm. of excess Congo red out of solution. The bag is partially collapsed to remove all the air and is then tied over the clamp (A). This is facilitated by a groove (C) on the outer surface of the clamp which allows the latex to be pulled in and make a watertight seal. The partially collapsed bag and clamp are placed in a glass ampoule (D) which has a splayed edge (E)

at one end and a nipple (F) to fit a plastic tube of 1 mm. internal bore at the other end. The excess latex (G) from the bag (B) is turned back over the splayed edge (E) of the ampoule (D) and tied with thread. The clamp and latex bag are fixed in the glass ampoule by means of ties which utilize the splayed edge as fixation points. The drug compartment (H) consists of the space between the Congo red bag and the glass ampoule. When the bag expands fluid is forced out through the nipple of the glass ampoule and is then carried by means of plastic connecting tubing to the desired organ. The water compartment consists of a second latex bag (I) which is tied over the splayed edge of the glass ampoule. This compartment is filled with distilled water either at the time the apparatus is assembled, or later, by means of a syringe and needle when the injector is buried in the animal. The water can now move under a constant osmotic gradient through the cellophane membrane into the Congo red compartment. More dye dissolves and a saturated solution is maintained; the movement of the stainless steel ball in the Congo red compartment helps mixing whilst the apparatus is in the animal. The Congo red bag expands and forces fluid containing the drug through the nipple. The whole arrangement is such that at least 2 ml. of water can diffuse into the bag. At the same time the Congo red solution will still be saturated, the bag will be full but not stretched and will fit neatly into the glass ampoule. Thus 2 ml. of drug will have been slowly and constantly forced through the nipple down the plastic tube to the desired organ. The time taken for this injection will, of course, depend on the area, thickness and permeability of the cellophane membrane, and these can be adjusted to suit the particular experiment. We have made slow injectors which will deliver 0·02 ml. per diem for 100 days, relatively fast injectors which will deliver 0·5 ml. per diem for 4 days, and various other injectors between these two extremes. In addition, injectors have been made in various sizes to suit the particular experiment and the size of the animal to be used. The apparatus illustrated is 6–7 cm. long and 1·4 cm. in diameter. This has been used in rabbits, dogs and large rats. A smaller apparatus capable of delivering only 0·8 ml. and measuring 1 cm. in diameter and 5 cm. in length has been made for particular use in the rat.

Assembly and sterilization of injector.

The injector is relatively easy to make and assemble so that only a few points need be discussed. All latex and glassware are carefully cleaned with three changes of boiling distilled water. Care must be taken to avoid air bubbles in the Congo red, water and drug compartments. We usually wash out the drug compartment two or three times with the same solution as will finally be used. This avoids errors due to adsorption of the drug on to glass and rubber which could be particularly important if low concentrations of drug are used. The drug compartment is then filled with the required solution. The nipple of the ampoule is immersed in the same solution and, with the water compartment empty and collapsed, the whole apparatus is heated to 80° C. for 15 minutes. This technique prevents any pressure from developing on either side of the cellophane membrane during sterilization. The apparatus, still immersed, is then cooled to room temperature and a sterile plug is used to close the nipple of the ampoule. The water compartment can now be filled and the apparatus weighed so that a check can be made on the final volume of fluid delivered. If the drug is not heat stable it can be introduced aseptically into the already heat sterilized apparatus. The external surface is sterilized by immersion in zephiran 1:1,000 for 15 minutes.

Preparation of plastic tube.

We have found polythene tube to be the best material from which to manufacture the needles and connecting tube for implantation. Polythene is relatively inert and causes very little tissue reaction. Commercial grades of polyvinyl chloride should not be used because various chemicals added during manufacture cause a severe local inflammatory reaction. We have also found surgical stainless steel hypodermic needles to be unsatisfactory; their rough internal surfaces makes them much more liable to become blocked with clots of blood. Polythene also has the advantage that it can be softened and drawn over a gas flame to any desired diameter. The exact size, shape, method of insertion and fixation of the polythene tube varies with the particular organ or tissue. When the correct polythene tube has been made it is completely filled with saline and the end which will connect with the injector is blocked with a stainless steel wire plug. At the time of operation this prevents blood from coming up the tube and blocking it with a long clot. The polythene tube is sterilized by immersion in saline for 15 minutes at 80° C. During the sterilization the tubing must be under slight tension otherwise it will become grossly distorted.

Operative technique.

All operations were carried out under surgically aseptic conditions and, in addition, pre- and post-operative penicillin cover was given. In the first stage an appropriate polythene tube is implanted and fixed into the particular organ or tissue to be studied. The wound is then closed with metal clips. In the second stage the injector is connected to the polythene tubing and buried in the subcutaneous tissues. A prior subcutaneous injection of 40 ml. of air lifts the skin and makes a convenient pocket. The injector is brought to body temperature by immersion in sterile saline. It is then placed in the subcutaneous pocket through an incision some distance away from where it will finally lie. The polythene tube is tested for patency—often minor blocks can be removed by pressure applied with a syringe and needle. The injector and tubing are connected and the wound closed with metal clips.

Performance of injector.

Depending on its size, the injector can deliver a particular total volume of fluid. This volume can be delivered at any desired rate by altering the area and thickness of the cellophane which separates the water and the Congo red solution. The rate of injection remains fairly constant whilst excess Congo red remains out of the solution. Tested in an incubator at 36° C., there is a slight variation (±6 p.c.) in the rate of injection from day to day. If blocked, the injector is capable of developing about 50 feet of water pressure. For all practical purposes this means that the resistance of the fine plastic tubing going to the organ does not alter the rate of injection. About 10 p.c. of all injectors

fail because the cellophane is broken or an air bubble comes in contact with one or other side of the cellophane.

Effect on animals.

The injector causes a mild inflammatory reaction in the subcutaneous tissues. Occasionally a serous or serosanguinous exudate develops, more so in the dog than the rat. The wound and subcutaneous tissues rarely become infected. We have not observed any untoward general effect on the animal's health when a small injector is buried in a large animal. Some of our experiments have required the use of an injector 7 cm. long and 1·4 cm. diameter and weighing 15 gm. These have been successfully buried in rats weighing 150–250 gm. Fig. 2 shows a rat weighing 220 gm. with such an injector *in situ*.

SUMMARY.

A simple long-term injector which is capable of delivering fluid at a constant rate for a long time has been described. The whole injector can be implanted in an animal which can thus receive a constant injection of a drug for several weeks without disturbance.

REFERENCE.

Bayliss, W. M. (1909): Proc. Roy. Soc. B., 81, p. 345.

LYSOGENICITY AND LYSIS PATTERNS IN THE SALMONELLAS

5. FURTHER TESTS WITH *S. BOVIS-MORBIFICANS*

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Atkinson *et al.* (1952) investigated 60 Australian strains of *S. bovis-morbificans* and found them all lysogenic, frequently carrying more than one type of phage. Complex lysis and lysogenicity patterns were obtained on which some of the strains were tentatively ascribed to four groups. Further investigation of the remaining strains revealed a new group, provisionally labelled group 6, which is described in this paper. This new group predominated among strains coming from Brisbane during the latter part of 1947 and early 1948 when an extensive outbreak of infantile gastro-enteritis, described by Mackerras and Mackerras (1949), was in progress.

METHODS.

Lysis tests were done according to the method for Vi phage typing of *S. typhi* (Craigie and Yen, 1938). Lysogenicity tests were done by growing the two required strains together on agar, extracting the mixed growth in broth, centrifuging the extract and testing it for lytic action on the strains from which it came.

RESULTS.

Lysis and lysogenicity tests.

Out of 60 heterogeneous strains of *S. bovis-morbificans* (numbered 1 to 60), representing human and animal strains from various parts of Australia, Atkinson *et al.* (1952) placed 22 strains in the provisional groups 1 to 4. The present work was carried out on the remaining 38 ungrouped strains (numbers 1, 2, 4, 6, 8, 9, 11, 12, 14, 16, 20 to 25, 27 to 31, 34, 37 to 44, 47, 51, 52, 55, 56, 58, 59, 60) of which strain 14 was recorded by Atkinson *et al.* (1952) as different from the strains in groups 1 to 4. Strain 14 has now therefore been allotted to the new provisional group 5 of which it has been selected as the type strain.

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To investigate the occurrence of group 5 strains, a preliminary screening of the other 37 ungrouped strains was made with phage BM14/18 which group 5 strains carried and to which they were resistant. The results of these tests are given in Table 1. No lysis was detected with 27 strains (1, 2, 6, 8, 12, 16, 21, 23, 24, 25, 27, 29, 34, 37, 38, 39, 41, 42, 43, 44, 47, 51, 52, 55, 56, 58, 60). Strains 30 and 31 were phage-infected and strain 20 grew poorly. With these three strains lysis tests could not be read and they were not investigated further. Strains 4, 9, 11, 22, 28, 40, 59 were lysed by phage BM14/18 and were set aside for further investigation later.

The 27 resistant strains were then tested for lysogenicity on strain 14 (group 5) and strain 18 (group 4). Strain 18 was included to detect phage of the BM14/18 type found in strains of group 5 and producing characteristic plaques with central secondary growth surrounded by irregular-edged clear lysis. All these strains were also tested together in batches of from seven to eleven strains in every combination of pairs. In these tests related strains should not show any lysogenic action when tested together.

The results of these tests indicated that group 5 strains were rare. Out of the 27 strains tested, only strain 44 resembled strain 14 on which it failed to show any lysogenic action. It also produced characteristic plaques of the BM14/18 type on strain 18. Strain 44 was therefore allotted to group 5 with strain 14. Among the other 26 strains tested, however, another new group, provisionally called group 6, also carrying phage of the BM14/18 type, became obvious. A strain of this new group 6 showed mutual lysogenic action with strain 14 and with strain 18. The group 6 strain and strain 14 lysed each other with small clear plaques. The group 6 strain was lysogenic on strain 18 producing strong lysis resembling that of phage BM14/18, while strain 18 lysed the group 6 strain with small clear plaques similar to those seen in the lysis of group 6 strains by strain 14. The group 6 strains were, of course, non-lysogenic among themselves. Thus, in the first batch of strains (1, 2, 6, 8, 21, 23, 27) tested in all combinations of pairs and on strains 14 and 18, strains 8, 21, 23 and 27 were mutually non-lysogenic, but with strain 14 and strain 18 showed the typical mutual lysogenicity described above for group 6. In the next batch, strains 34, 37, 41, 43, 47, 51 and 52 were mutually non-lysogenic and gave with strains 14 and 18 the typical lysogenicity pattern of group 6. Further, to confirm the similarity of strains 8, 21, 23, 27, 34, 37, 41, 43, 47, 51 and 52, they were all tested together for lysogenic action in every combination of pairs. No lysis was detected and these strains were considered to be identical. They were allotted to the new group 6 and strain 8 was selected as the type strain. Strains 55, 56 and 58 tested together and with strains 8, 14 and 18 also gave the reactions of group 6. Strains 12, 24, 25, 29, 38, 39, 42, 60 tested together and with strains 8, 14 and 18 gave heterogeneous reactions and none of them resembled the group 6 strains. Thus, out of the 38 strains in this investigation, two (strains

14 and 44) were placed in group 5, 14 (strains 8, 21, 23, 27, 34, 37, 41, 43, 47, 51, 52, 55, 56, 58) in group 6, and the remainder were ungrouped. The grouping of these 38 strains, together with the place and date of isolation of each are given in Table 1.

Table 1 shows the heterogeneous origins of the 38 strains. However, an examination of the sources of the 14 strains placed in group 6 showed that they were not a heterogeneous group. Twelve of them came from Brisbane during the period from August, 1947, to March, 1948, when an extensive epidemic of

TABLE 1.

Grouping of 38 heterogeneous ungrouped strains of *S. bovis-morbificans*.

Strain no.	Place and date of isolation	Lysis with phage BM14/18	Grouping
[1]1	Brisbane, Q. 2/48	–	ungrouped
2	Melbourne, Vic. 4/47	–	ungrouped
4	Sydney, N.S.W. 2/49	+	ungrouped
6	Sydney, N.S.W. 11/49	–	ungrouped
[1]8	Brisbane, Q. 9/47	–	group 6
9	Adelaide, S. Aus. 9/48	+	ungrouped
11	Sydney, N.S.W. 9/50	+	ungrouped
12	Adelaide, S. Aus. 3/48	–	ungrouped
14	Sydney, N.S.W. 10/50	–	group 5
16	Adelaide, S. Aus. 7/49	–	ungrouped
[1]20	Brisbane, Q. 9/47	0	growth poor tests unreadable
[1]21	Brisbane, Q. 11/47	–	group 6
22	Sydney, N.S.W. 4/49	+	ungrouped
23	Adelaide, S. Aus. 2/49	–	group 6
24	Adelaide, S. Aus. 5/49	–	ungrouped
[1]25	Brisbane, Q. 9/47	–	ungrouped
[1]27	Brisbane, Q. 3/48	–	group 6
28	Adelaide, S. Aus. 4/47	+	ungrouped
29	Brisbane, Q. 8/49	–	ungrouped
30	Brisbane, Q. 5/48	0	phage infected
31	Sydney, N.S.W. 6/50	0	phage infected
[1]34	Brisbane, Q. 12/47	–	group 6
[1]37	Brisbane, Q. 9/47	–	group 6
38	Adelaide, S. Aus. 4/48	–	ungrouped
39	New Zealand, 6/48	–	ungrouped
40	Adelaide, S. Aus. 2/48	+	ungrouped
[1]41	Brisbane, Q. 2/48	–	group 6
42	Sydney, N.S.W. 11/49	–	ungrouped
[1]43	Brisbane, Q. 9/47	–	group 6
44	Brisbane, Q. 5/51	–	group 5
[1]47	Brisbane, Q. 11/47	–	group 6
51	Adelaide, S. Aus. 3/51	–	group 6
[1]52	Brisbane, Q. 9/47	–	group 6
[1]55	Brisbane, Q. 11/47	–	group 6
[1]56	Brisbane, Q. 3/48	–	group 6
[1]58	Brisbane, Q. 3/48	–	group 6
59	Adelaide, S. Aus. 8/48	+	ungrouped
60	Adelaide, S. Aus. 5/48	–	ungrouped

[1] Brisbane strains of the epidemic period. 0 = no result obtainable.
The date is given as the month and year thus, 2/48 for Feb., 1948.

infantile gastro-enteritis occurred (Mackerras and Mackerras, 1949). Hundreds of Brisbane strains of *S. bovis-morbificans* were typed in this laboratory during that period and 15 of them (strains 1, 8, 20, 21, 25, 27, 34, 37, 41, 43, 47, 52, 55, 56 and 58) were included in the present investigation and are marked 1 in Table 1, for easy recognition. Table 1 shows that all of these except three (strain 20, which grew poorly and was not tested, and strains 1 and 25 ungrouped) were placed in group 6. Strain 1 was an animal strain. Thus group 6 strains of *S. bovis-morbificans* were well represented among the strains of the Brisbane epidemic period. Only two other group 6 strains (23 and 51) appear in Table 1 and these came from Adelaide.

As a contrast to the 38 heterogeneous strains in Table 1, a further 20 strains (numbered 61 to 80) were selected from Brisbane strains from different patients sent by Dr. I. M. Mackerras, of the Queensland Institute of Medical Research, during September, 1947. These strains, except strain 65 which failed to grow well, were tested for lysis with phage BM14/18 and for lysogenicity with strains 8, 14 and 18. They were also tested together in all combinations of pairs in various batches of which a typical test contained strains 63, 66, 67, 68, 69, 70, 71 and 72. Strains 75 and 80 failed to give the reactions of group 6. The other 17 strains (61, 62, 63, 64, 66, 67, 68, 69, 70, 71, 72, 73, 74, 76, 77,

TABLE 2.

Grouping of 20 different human strains of *S. bovis-morbificans* from Brisbane during the epidemic period.

Strain no.	Place and date of isolation	Lysis with phage BM14/18	Grouping
[1]61	Brisbane, Q. 9/47	—	group 6
62	Brisbane, Q. 9/47	—	group 6
63	Brisbane, Q. 9/47	—	group 6
[1]64	Brisbane, Q. 9/47	—	group 6
65	Brisbane, Q. 9/47	0	growth poor tests unreadable
[1]66	Brisbane, Q. 9/47	—	group 6
67	Brisbane, Q. 9/47	—	group 6
68	Brisbane, Q. 9/47	—	group 6
69	Brisbane, Q. 9/47	—	group 6
70	Brisbane, Q. 9/47	—	group 6
71	Brisbane, Q. 9/47	—	group 6
72	Brisbane, Q. 9/47	—	group 6
73	Brisbane, Q. 9/47	—	group 6
74	Brisbane, Q. 9/47	—	group 6
[1]75	Brisbane, Q. 9/47	—	ungrouped
[1]76	Brisbane, Q. 9/47	—	group 6
77	Brisbane, Q. 9/47	—	group 6
78	Brisbane, Q. 9/47	—	group 6
[1]79	Brisbane, Q. 9/47	—	group 6
80	Brisbane, Q. 9/47	—	ungrouped

0 = no result obtainable.

[1] Strains from post-mortem material.

78, 79), however, belonged to group 6. They were mutually non-lysogenic in the batches tested, they failed to lyse strain 8 (the type strain of group 6), and they gave the typical group 6 lysogenicity patterns with strains 14 and 18. The groupings of these strains are given in Table 2.

Thus, out of the 20 Brisbane strains from the epidemic period, one grew poorly and was not tested, two were ungrouped and 17 belonged to group 6.

Combining the results in Tables 1 and 2 of tests on all Brisbane strains from the epidemic period, 34 strains were tested of which 27 were placed in group 6, two could not be tested on account of poor growth and five were ungrouped. Thus the majority of these strains were placed in group 6.

Characteristics of group 6.

Strains of this provisional group were resistant to phage BM14/18 and carried at least two kinds of phage, one of which appeared to be identical with the phage BM14/18, also carried by group 5 strains. The second phage produced very small clear plaques on strain 14 (group 5) and seemed to be characteristic of group 6, distinguishing it from group 5. Group 6 strains also lysed strain 18 (group 4) with characteristic lysis typical of phage of the BM14/18 type. Group 6 strains were lysed by strain 14 (group 5) or strain 18 (group 4) with small clear plaques. Thus group 6 strains were mutually lysogenic with strain 14 and strain 18, producing recognizable lysis patterns. They were non-lysogenic with strain 8 (group 6). The reactions of groups 4, 5 and 6 are given in Table 3.

TABLE 3.

Reactions of groups 4, 5 and 6 of *S. bovis-morbificans*.

Group no.	Lysis with phage BM14/18	Lysogenicity on		
		Strain 18 (group 4)	Strain 14 (group 5)	Strain 8 (group 6)
4	+	−	−	+
5	−	+	−	+
6	−	+	+	−

To demonstrate that group 6 differed from groups 1, 2 and 3, the type strains of these groups, strain 7 (group 1), strain 17 (group 2) and strain 45 (group 3) were tested for lysogenicity with strain 8 (group 6). Strain 7 lysed strain 8, strain 17 and strain 8 were mutually lysogenic and strain 45 and strain 8 were also mutually lysogenic. The type strain 8 of group 6 was therefore different from the type strains of groups 1, 2 and 3.

DISCUSSION.

The new provisional group 6 of *S. bovis-morbificans* defined by lysis and lysogenicity patterns, contained the largest number of strains so far allotted to any *S. bovis-morbificans* group. This apparent predominance of group 6 was due to the inclusion in the tests of a considerable number of strains from Brisbane during the epidemic of 1947-48. Most of these strains were placed in group 6, but very few strains from other places or from Brisbane at other periods belonged to group 6. These other heterogeneous strains, not belonging to group 6, have not yet been grouped but are under investigation. They appeared to form several groups. They showed no homogeneity like the Brisbane epidemic strains. More than 200 of these strains are available here and will be grouped. From these results the epidemiological significance of group 6 should become more apparent.

The validity of group 6 as compared with the other groups was established by positive lysogenicity tests with the type strains of groups 1, 2, 3, 4 and 5. Group 6 was related to group 5 by carriage of phage like BM14/18. It differed from group 5 in carrying a second phage lysing group 5 strains and was itself sensitive to the second phage of group 5 strains. Groups 5 and 6 were similar therefore in carrying phage like BM14/18, but each also carried a second phage, typical of the group, by which each could be distinguished. Strain 14, described ungrouped by Atkinson *et al.* (1952), who showed it to be different from strains of groups 1, 2, 3 and 4, was allotted to the new provisional group 5. Its characteristics were adequately covered here and it contained only two strains (14 and 44). Thus the heterogeneity of strains of *S. bovis-morbificans*, when investigated for lysogenicity, and the multiplicity of phages carried, as suggested by Atkinson *et al.* (1952), was further confirmed in the present work.

SUMMARY.

By lysis and lysogenicity tests on 38 heterogeneous strains of *S. bovis-morbificans*, two new provisional groups, numbered 5 and 6, were found. These groups each carried at least two kinds of phage, one of which seemed identical with phage BM14/18 and the other was characteristic of the group. This second phage was responsible for mutual lysogenicity between groups 5 and 6.

Of the 38 strains tested, two were placed in group 5 and 14 in group 6.

Twelve of the group 6 strains came from Brisbane between August, 1947, and March, 1948, when an extensive outbreak of infantile gastro-enteritis was in progress. A further 20 Brisbane strains from this epidemic were selected and tested and 17 were placed in group 6. This group was thus well represented among the Brisbane epidemic strains.

REFERENCES.

Atkinson, N., Geytenbeek, H., Swann, M. C., and Wallaston, J. M. (1952): Austral. J. exp. Biol., 30, p. 333.

Craigie, J. and Yen, C. H. (1938): Canad. pub. Health J., 29, p. 448.

Mackerras, I. M. and Mackerras, M. J. (1949): J. Hyg., 47, p. 166.

THE FUNCTIONAL ACTIVITY OF THYROID NODULES IN AN AREA OF ENDEMIC GOITRE

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Goitre is endemic in Northern Tasmania and commonly occurs as the nodular type. The nodules are of both the colloid and parenchymatous variety. The purpose of this work was to investigate the functional activity, as assessed by radio-autographs, of nodules from clinically non-toxic cases.

Material and Methods.

Thyroid glands were removed from nineteen patients with non-toxic nodular goitre. All patients had received a thorough clinical examination, including radioactive iodine tracer tests and estimations of the plasma protein-bound iodine and of the 48-hour plasma protein-bound radioactive iodine. Operation was performed either for relief of pressure or for cosmetic reasons. No iodine or thiouracil was given pre-operatively.

Each patient received intravenously some 24 hours prior to operation approximately 150 micro-curies of radioactive iodine (half life of 8 days). The quantity of inorganic iodine in this dose is infinitesimal and does not produce any recognisable change within the gland.

After operation all glands were examined macroscopically and sectioned at 2 cm. intervals. Sections of nodules with surrounding normal paranodular tissue were taken and divided into two portions. One portion was reserved for the usual histological examination and from the other paraffin sections were prepared by a rapid method as follows:

(1) Fixation for two hours at 60° C. in 4 p.c. formol saline previously neutralized over calcium carbonate.

(2) 1 hour in 70 p.c. ethyl alcohol.

(3) 2 hours in absolute alcohol (1 change).

(4) 30 minutes in purified benzol.

(5) Vacuum embedding in paraffin for 30 minutes at 10 lbs./sq. in.

Sections were prepared and mounted on the emulsion side of extra-contrasty Kodak lantern plates and stored in the dark for a suitable interval. They were then developed, fixed, and stained with haematoxylin and eosin. The method is similar to that described by Evans.

The varied content of radioactive iodine in the different types of thyroid tissue is shown by the degree of blackening of the plate caused by the radio-iodine in the overlying tissue. Because of its affinity for haematoxylin, the emulsion in the background tends to overstain, but this can in part be prevented by staining with metanil yellow prior to the haematoxylin. The resulting stain leaves the colloid orange-yellow and the emulsion a brownish-yellow. Both methods were used.

As a further means of trying to assess the relative degrees of functional activity of nodular and paranodular tissue, frozen sections of fresh thyroid were incubated at 37° C. for 30 minutes with 2, 3, 5 triphenyltetrazolium chloride in normal saline buffered to pH 7·4.

Under such conditions the enzyme activity of the cells causes the deposition of a red insoluble formazan on the sections.

Definitions. (1) A nodule or "adenoma." A nodule is any circumscribed discrete mass whose naked eye structure is different from surrounding normal thyroid tissue. Only two types are discussed here, namely the colloid adenoma which is cystic and succulent in appearance, and the parenchymatous adenoma, which is solid and greyish white.

(2) I.131—radioactive iodine (half life 8 days).

(3) P.B.I.—blood plasma protein-bound iodine expressed in gamma/100 mls.

(4) P.B.I.$_{(131)}$—blood plasma protein-bound radioactive iodine expressed as p.c. of dose administered per litre.

(5) Tracer uptake. This refers to the rate of uptake of I_{131} by the thyroid gland in the living subject. For the purposes of this paper the uptake will be expressed either as "normal" or "toxic."

RESULTS.

Triphenyltetrazolium chloride technique. This substance was rapidly converted to the red insoluble formazan on incubation at 37° C. for 30 minutes with frozen sections of thyroid tissue. The formazan was deposited as red crystals on the surface of the epithelium lining the follicles. It was hoped that under standard conditions the intensity of the deposit might correspond to the degree of epithelial hyperplasia, but, unfortunately, no satisfactory correlation was found between the histological structure and the quantity of the deposit. It was concluded that this method offered no helpful information.

Affinity for radioactive iodine shown by parenchymatous nodules with a predominantly micro-follicular structure. Six nodules each from different glands were examined. All glands were of the non-toxic nodular variety. Five of the nodules showed little or no uptake compared with the paranodular tissue, which it is probably reasonable to accept as normal. All showed a slight degree of central degeneration. In one instance the degenerate follicles retained a small amount of radioactive iodine. The sixth nodule had a moderate affinity for I_{131}, which, however, was less than that of the normal paranodular tissue. This nodule did not show central degenerative changes, the cells were taller than those of the other nodules and acini were more plentiful.

Affinity for radioactive iodine shown by colloid nodules. Thirteen nodules each from different non-toxic glands were compared with normal paranodular tissue for I_{131} uptake. All colloid nodules took up I_{131} but usually to a less degree than normal tissues. The amount of I_{131} taken up appears to be directly related to the degree of epithelial hyperplasia within the nodule, follicles with hyperplastic changes retaining more I_{131} than simple follicles. An exception to this rule was seen only once; a group of follicles lined by cuboidal epithelium contained much more I_{131} than a neighbouring group with hyperplastic epithelium.

Although all the patients were clinically completely free of toxic manifestations, one, Mrs. C.L., aged 33, showed an excessive uptake of tracer. This was regarded as being of "toxic" level. The P.B.I. and the 48-hour plasma P.B.I.$_{131}$ were normal. In the excised gland there were two nodules and one of these, hyperplastic on ordinary histological examination, showed a much greater affinity for I_{131} than is present in normal parenchyma (Fig. 1).

Fig. 1. This shows radioactive iodine uptake (black areas) in a nodule showing hyperplastic changes.

Discussion on the affinity of thyroid nodules for radioactive iodine. The results show that the solid adenoma-like nodules have a very much smaller affinity for radioactive iodine than normal thyroid tissue. This fact, together with the histological evidence of cellular inactivity, supports the view that they are functionally inert and play little part in the iodine economy of the gland.

The colloid type of nodule shows slightly more affinity for I_{131}, but this is generally less than is shown by the paranodular tissue. The degree of uptake

is slightly more variable in the colloid nodule than in the parenchymatous ones, and the content of I_{131} may be actually greater than in the surrounding normal areas. When this occurs the nodule shows histological evidence of proliferative and hyperplastic changes. An extreme example of this was seen in the gland from Mrs. C.L. mentioned above, and the findings in her case suggest that the high uptake of radioactive iodine noted pre-operatively was due to a developing activity in this nodule. This might have been merely a temporary phase of activity, but on the other hand this nodule may have been the beginning of a toxic lesion, and the tracer test may in fact provide the earliest evidence of the onset of toxic change.

The results are substantially in agreement with those of other authors. Leblond *et al.* (1946) carried out radioiodine fractionation studies on pathologic thyroid tissue removed from 7 patients with varied clinical types of goitre. The data indicated that the cells of nodular adenomatous thyroid tissue showed consistently less avidity for iodine and produced less tyrosine and diiodotyrosine than the group of thyroid cells in the surrounding thyroid tissue. The difference was most marked in one so-called "foetal adenoma." Rawson *et al.* (1948) measured the radioiodine uptake of a variety of thyroid nodules. The quantity of radioactivity in each specimen was then expressed as a per cent. per gram of tissue of the radioiodine administered. They described three nodules of microfollicular variety which showed a greater avidity for iodine than normal tissues. This has not been our experience, although this paper substantiates the general principle that the capacity of thyroid nodules to absorb iodine can be correlated with the degree of differentiation observed on histological examination.

The results obtained from autoradiographs have to be interpreted with caution. Admittedly, they are influenced by the affinity of the tissue for radioactive iodine, but the amount present in the section is an index only of the amount present at the time when the tissue was fixed. Some loss may be caused by the technical processes of fixation, dehydration and embedding, but this is probably the same for all the tissues and thus is not too misleading. The correlation with the results of the tracer tests is also complicated by the difficulty of knowing how rapidly the uptake iodine is altered and released again into the circulation. None the less, it seems reasonable to deduce from the findings given that the parenchymatous nodules are of low functional activity, and that the colloid nodules are the ones that are likely to become endocrinologically active, although in the clinically non-toxic case, they also are generally inactive. Possibly the tracer test gives evidence of a change to the toxic state before any of the usual clinical symptoms appear.

SUMMARY.

Autoradiographic studies on nodules from 19 different goitrous thyroids from non-toxic patients in an area of endemic goitre show that:

Parenchymatous nodules take up practically none of the radio iodine ingested 24 hours earlier.

Colloid nodules are more active than parenchymatous ones, but are mainly less active than normal thyroid tissue.

A colloid nodule may hyperfunction without, however, necessarily producing the clinical picture of toxic adenoma.

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REFERENCES.

Simmel, Fitzgerald, and Godwin (1951): Stain Tech., 26, p. 25.
Dobyns and Lennon (1948): J. clin. Endocrin., 8, p. 737.
Leblond, Puppel, Riley, Radike, Curtis (1946): J. lab. clin. Med., 31, p. 484.
Evans (1947): Radiology, 49, p. 206.
Rawson, McArthur, Dobyns, Fluharty and Cope (1948): West. J. Surg., 56, p. 82.
Forbes (1951): Proc. Soc. Roy. Austral. Coll. Phys., 6, p. 116.

PORPHYRINS WITH FORMYL GROUPS V[1]

ISOLATION OF PORPHYRIN *a* FROM HEART MUSCLE AND DETERMINATION OF ITS HAEM *a* CONTENT

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The porphyrin which is the iron-free prosthetic group of cytochrome a_3 (cytochrome oxidase), of cytochrome *a* and probably of cytochrome a_1 of bacteria, but not of cytochrome a_2 (Barrett and Lemberg, 1954), was first studied by Rawlinson and Hale (1949) and was called porphyrin *a*. Further work on its properties has been carried out by Rawlinson *et al.* (1949), Rimington *et al.* (1949), Lemberg *et al.* (1949), Lemberg and Falk (1951), Falk and Rimington (1952), Granick (1952), Dannenberg and Kiese (1952), Kiese (1952), and Lemberg (1953, 1955).

The haemin, first studied by Negelein (1933) has been obtained crystalline and called cytohaemin by Warburg and Gewitz (1951), but neither the method of its preparation, nor its spectroscopic properties or the porphyrins obtained from it have as yet been described. Lemberg (1953) has shown that by a new procedure porphyrin *a* could be isolated from heart muscle in excellent yield, and that this porphyrin differed from all previous preparations in its absorption spectrum. It was concluded that the porphyrin *a* obtained by the previous procedure was either altered or still accompanied by other porphyrins.

In the present paper we describe this later method and also discuss in detail the advantages and disadvantages of various purification procedures developed both in our laboratory and elsewhere. The first aim was to develop a method by which the porphyrin *a* could be extracted practically quantitatively, without alteration, and with complete separation from other porphyrins. This aim has been achieved. It is now possible to determine the porphyrin *a* content of heart muscle spectrophotometrically provided that the specific extinction of porphyrin *a* at a chosen wavelength is known. This determination can be made without the necessity for complete separation of porphyrin *a* from colourless

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[1]II, cf. R. Lemberg and J. Parker. This journal, 30, 163 (1952). III, cf. R. Lemberg (1953). IV, cf. R. Lemberg (1955).

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impurities which cannot be done quantitatively. The best method (method B_3, see below) for the purification of porphyrin *a*, described in this paper, produces porphyrin *a* of sufficient spectroscopic purity in very high yield. However, the final ether solutions of the porphyrin still contain colourless impurities. The further purification will be described in a separate paper (Lemberg and Stewart, 1955). The specific extinction of our best preparations consistently reaches the same high value, E, 0·1 p.c., 1 cm. at 558 mμ in ether solution (ϵ_{sp}^{558}) = 20. This extinction is used to calculate the yield in mg. per kg. of heart muscle.

Our method of treatment yields practically only protoporphyrin and porphyrin *a*, and only a trace of another porphyrin, cryptoporphyrin *a*, which closely resembles chlorocruoroporphyrin. Other methods have been found to give substantial amounts of cryptoporphyrins, partly derived from haemin *a* and partly from protoporphyrin. A cryptoporphyrin was first isolated from pigeon breast muscle by Negelein (1932). In a separate paper the isolation of cryptoporphyrin *a* and of some other cryptoporphyrins in crystalline form will be reported (Parker, 1955; cf. also Lemberg, 1953).

EXPERIMENTAL.

Method of measurement. Spectrophotometric measurements were carried out with a Hilger Uvispek Photoelectric spectrophotometer. The absorption spectrum of porphyrin *a* in ether shows four bands in the visible part of the spectrum: I, 646; II, 583; III, 558; IV, 517 mμ (III>II>IV>I). Band II has a slight shoulder towards the longer wavelength side which appears as a distinct band in a small dispersion spectroscope. The amount of porphyrin *a* is calculated with the formula:

$$\text{mg. porphyrin } a = \epsilon_{558} \times v/20,$$

where ϵ_{558} mμ is the extinction coefficient (D/l. in cm.) at the maximum of band III, v the volume of the solution in ml., and 20 the specific extinction coefficient found for our purest preparation of porphyrin *a* at 558 mμ. As a criterion of purity we use the ratio of the extinctions at the absorption maxima of band III and band IV in ether solution (R III/IV=D_{558} mμ/D_{517} mμ). This is a sensitive criterion, since R III/IV is 2·00 for pure porphyrin *a*, but much smaller for the other porphyrins which are extracted from heart muscle, such as protoporphyrin (0·23) and cryptoporphyrin (1·10). Moreover, secondary alteration of porphyrin *a* has often been found to decrease, but never to increase R III/IV.

Methods of preparation. Altogether more than 100 experiments were carried out to arrive at optimal conditions for the isolation of porphyrin *a*. This was only gradually achieved and in this section the four main methods are described which we successively adopted. These four methods are:

Method A: Extraction of haemins from wet sediment of homogenate with acetone-HCl. Porphyrin *a* extracted from ether by 20 p.c. HCl.

Method B_1: Extraction of haemins from frozen and thawed muscle with acetone-HCl, adjusting water content to 20 p.c. Porphyrin *a* extracted from ether by 20 p.c. HCl.

Method B_2: Frozen and thawed muscle pre-extracted with neutral 80 p.c. acetone, haemins extracted with acetone-HCl containing 20 p.c. of water. Porphyrin *a* measured before and after extraction from ether by 20 p.c. HCl.

Method B_3: As B_2, but modified method of removal of iron. Porphyrin *a* not extracted by 20 p.c. HCl, but measured before and after chromatography on cellulose powder column.

TABLE 1.

Yields of porphyrin *a* from ox heart muscle[1].

Method[2]	Preparation	No. of measurements	mg. per kg. dry wt.[3]		mg. per kg. fresh wt.[3]		R III/IV	
			before[4, 5] HCl	after[4, 5] HCl (or chromatography)	before	after HCl (or chromatography)	before	after HCl (or chromatography)
A	A 29-65	11	[(57)]	[46]	(16·4)	13·1±2·9	—	1·88±0·20
B_1	A 74-78	4	[(80)]	[64]	(22·5)	18·0±2·2	—	1·88±0·05
B_2	A 87-101	10	77	63±13	21·6	17·7	1·87±0·18	1·96±0·10
B_3	A 102-119	12	103±11	75[6]	28·8±4·2	21·4[6]	1·86±0·07	2·04±0·04[6]

[1] All values calculated from $\epsilon_{558\ m\mu}$ using $\epsilon_{sp}^{558\ m\mu} = 20 \cdot 0$.

[2] See text.

[3] In [ ] if calculated on basis of dry wt. forming 28 p.c. of wet wt.

[4] In ( ) if calculated on basis of 80 p.c. yield in extraction with 20 p.c. hydrochloric acid.

[5] Before HCl: in ether after removal of other porphyrins with 8 p.c. HCl.

[6] After chromatography on cellulose powder.

Various modifications which were tried and which were found of no advantage are briefly described in a later section.

The procedures can be divided broadly into four steps: (1) the preparation of the heart muscle tissue for extraction of the haemins, (2) the extraction of the haemins, (3) their conversion to porphyrins, and (4) the separation of the porphyrins.

(1) *Preparation of heart muscle tissue.* Ox hearts were used for these experiments. The hearts were received within one to four hours after slaughter. After trimming and removing as much fat as possible, they were minced, and the mince washed four times with tap water and once with distilled water. The washed mince was frozen at −15° C. It could be kept in this state for several weeks without detriment. The frozen mince was allowed to thaw overnight at 4° C. before extraction.

In experiments A29–78 (Table 1, method A), the wet weights of the fresh mince were determined, and the yield in terms of mg. porphyrin *a* per kg. of dry weight was calculated on the basis of the average dry weight of the mince. In experiments A87–101 (method B_1) the wet and (in an aliquot) dry weights of the washed, frozen and thawed mince were determined. The wet weight of the washed mince can differ considerably from that of the fresh mince. For this reason, the yield per kg. of fresh heart muscle is here calculated on the basis of the dry weight of the washed mince. This decreases only slightly during the washing, causing yields by method B_1 to be slightly too high. In experiments A102–117 (methods B_2 and B_3) wet and dry weights of the fresh mince were determined.

In the earlier experiments (A29–65, Table 1) 500 gm. of the thawed mince were homogenised in a Waring Blendor for 5 minutes in 100 gm. fractions suspended in 150 ml. ice-cold water. The homogenates were then centrifuged at 1,500 r.p.m. for 20–30 minutes, and the sediment used for the extraction of the haemins. When this process was used, no water was added to the acetone-hydrochloric acid mixture used for extraction; the sediment contained sufficient water to bring the water content to about 20 p.c. In later experiments (method B, from A74 onwards), the frozen and thawed mince was directly extracted with acetone-HCl, adjusting the water content of the extract to 20 p.c. (control when necessary by density).

Extraction with acetone-hydrochloric acid of low water content gives poor results (see below), nor could a mince once extracted with neutral acetone or alcohol be later satisfactorily extracted with the 80 p.c. acetone-hydrochloric acid mixture. A pre-extraction with neutral 80 p.c. acetone-20 p.c. water was, however, possible, and this was applied in the later experiments (from A87 onwards, methods B_2 and B_3 of Table 1). This pre-extraction does not so much increase yield and purity, as remove emulsifying substances which can greatly slow down later fractionations.

(2) *Extraction of the haemins.* Two litres of an 80 p.c. acetone-20 p.c. water mixture containing 0·7 p.c. (w/v.) hydrochloric acid were used for the extraction of 500 gm. of fresh tissue or the homogenate sediment derived therefrom. After 16 hours at 4° C. the tissue is practically colourless and further extracts yield little haemin. For analytical purposes it is necessary to use at least 400 ml. of acetone-HCl per 100 gm. of fresh tissue, and the analysis can be carried out satisfactorily with as little as 5 gm. of fresh heart (see below). For preparative purposes 2 l. instead of 4 l. per kg. of fresh muscle can be used, and the extraction can be carried out at −15° C. instead of at 4° C. without serious loss of porphyrin *a*.

The water content of the filtrate is now adjusted to about 30 p.c. (sp.g.=0·895) and fat is frozen out overnight at −15° C., and filtered off in the cold. This yellow fat is practically free from haemin unless the water concentration is above 30 p.c.

The haemins are now transferred from acetone into ether by addition of 1 p.c. aqueous hydrochloric acid, to which a small amount of ferrous sulphate may be added (see below). The aqueous phase is twice re-extracted with ether; it contains flavins, traces of free porphyrins, and occasionally biliverdin. The ether is finally washed thoroughly with 1 p.c. hydrochloric acid to remove acetone and a white substance which collects in the interphase. The removal of the greater part of this emulsifying agent at this stage can usually be carried out without centrifugation, and this greatly facilitates later fractionations. Another advantage is that the evaporation of large volumes of acetone hydrochloric acid solutions is avoided. Even if this is carried out at room temperature *in vacuo*, evidence for partial destruction and alteration of haemin *a* during the evaporation has been found (see below). Finally, a considerable amount of haemin can be removed by crystallization from the concentrated ether solution on standing at −15° C. This beautifully crystalline haemin consists mainly of protohaemin with 3 p.c. haemin *a*. Since it contributes less than 2 p.c. of the total porphyrin *a*, it was usually discarded. The ether mother liquor contained the bulk of haemin *a* together with an approximately equal amount of protohaemin.

(3) *The removal of iron from the haemins.* Iron is removed from haemin *a* somewhat more readily than from protohaemin, but porphyrin *a* is not very stable in hot glacial acetic acid. The ferrous acetate-hydrochloric acid method was therefore modified. The ether extract of the haemins was evaporated *in vacuo* and the residue dissolved in glacial acetic acid, using about 50 ml. for the haemins from 500 gm. muscle mince. Under a current of carbon dioxide the solution was at once rapidly heated in a boiling water bath to 75–80° C. As soon as this temperature was reached, 0·3 ml. of freshly prepared near-saturated colourless ferrous acetate solution and 2 ml. conc. hydrochloric acid were added in rapid succession. The colour turned rapidly from brown to blue-green. The solution was immediately removed from the hot bath, rapidly cooled under running cold water and poured into a separating funnel containing ether over dilute aqueous sodium acetate solution, mixing at once vigorously. Two further extractions with small volumes of ether exhaust the porphyrins in the aqueous phase. This process splits the haemins completely in less than one minute from the beginning of the heating to the extraction of the porphyrins with ether, and forms no ether-insoluble porphyrin. The colour of the ether solution is raspberry red, rather than brownish purple as with other porphyrins. Its spectrum shows, besides the four bands of protoporphyrin (at about 631, 580, 538, and 505 mμ), two additional bands at 645 and 556 mμ which are mainly due to porphyrin *a*, its two other bands being masked by those of protoporphyrin. This method was used in methods A, B_1 and B_2 (Table 1).

In later experiments with haemin *a* reconstituted from porphyrin *a*, it was found that an excess of ferrous acetate during the removal of iron from the

haemin causes a partial alteration of porphyrin *a*, indicated by the lowering of R III/IV (see below). Since it is difficult to control the amount of ferrous acetate, a mixture of the stable ferrous sulphate with sodium acetate was added to the acetic acid solution of the haemin. After systematic trials (see below) the following method was adopted. 2·0 ml. of a slightly acidified solution of 5 p.c. (w/v.) $FeSO_4 \cdot 7H_2O$ were added at 80° C. immediately followed by 2 ml. of conc. hydrochloric acid. The solution was then brought into ether with rapid cooling as described above. The molar ratios of haemin: Fe^{2+}:HCl are roughly 1:10:500. This method was used for experiments A106–119 (Methods B_3 of Table 1).

(4) *Separation of the porphyrins.* Most of the acetic acid is removed from the ether solution by several washings with distilled water. Emulsions which may form at this stage are broken by the addition of small amounts of saturated ammonium sulphate solution. Protoporphyrin is then extracted with 2–3 p.c. (w/v.) hydrochloric acid and the last traces with 4 p.c. HCl until its absorption band at 538 mμ can no longer be discovered in the remaining ether solution. The porphyrin driven back from the 2–3 p.c. HCl extracts into ether with sodium acetate has the typical absorption spectrum of protoporphyrin (I, 631; II, 576; III, 538; IV, 503 mμ; IV>III>II>I) without any band at 555 mμ. The porphyrin obtained from the 4 p.c. HCl extract is also predominantly protoporphyrin, but the ether solution shows a faint band at 555 mμ in addition to the 538 mμ band III of protoporphyrin. More of this porphyrin (cryptoporphyrin *a*) is extracted by 8 p.c. (w/v.) HCl. Its ether solution shows absorption bands at I, 642; II, 580; III, 555; IV, 513 mμ; (III>IV>II>I). Superficially, the spectrum resembles that of a mixture of porphyrin *a* with protoporphyrin, and it has therefore been overlooked by other workers. The complete absence of band III of protoporphyrin at 538 mμ, however, together with the approximately equal intensity of bands III and IV militated against this assumption. It could be shown, first by spectrophotometric investigations (Lemberg and Parker, 1955) and later by isolation of crystalline cryptoporphyrin *a* ester (Parker, 1955), that this fraction contained a porphyrin different from both protoporphyrin and porphyrin *a*. In our preparation little cryptoporphyrin is formed, less than 10 p.c. of the porphyrin *a*. It is noticeable that no porphyrin *a* is extracted by 8 p.c. HCl at this stage of the procedure.

Porphyrin *a* is left in the ether phase after extraction with 8 p.c. HCl. The raspberry-red solution, which shows little red-green dichroism, has the following absorption spectrum in a small dispersion spectroscope: I, 646; II, 594 . . . 580; III, 558–7; IV, 517–6 mμ (III>II>IV>I). In the spectrophotometer the band at 594 mμ appears only as a faint shoulder on the band whose maximum is found at 583 mμ. In the earlier experiments (up to A78, methods A and B_1 of Table 1), this ether solution still contained variable amounts of a yellow substance, the presence of which lowered R III/IV to 1·5–1·7. Such solutions

were not considered pure enough for spectrophotometric determination of their porphyrin *a* content. Porphyrin *a* was therefore extracted from ether by 20–25 p.c. (w/v.) hydrochloric acid, giving a blue-green acid solution and leaving the yellow substance in the ether. After repeated washing of the HCl extracts with ether, the porphyrin was driven into fresh ether by dilution of the acid to about 10 p.c. followed by gradual neutralization with solid sodium bicarbonate. After this R III/IV was above 1·8 and occasionally reached 2·0. These solutions were then used for spectrophotometric measurement (methods A and B_1 of Table 1) as described under "Method of Measurement."

The procedure of extracting porphyrin *a* with strong aqueous hydrochloric acid solutions has several disadvantages. In contrast to the crude porphyrin *a* obtained by the method of Falk and Rimington (1952) (see below), absorption spectrum and extinction of our porphyrin *a*, even at this stage of purity, is quite stable in 25 p.c. (w/v.) HCl, although extracts in 28 p.c. and 30 p.c. HCl show a slightly lower R III/IV. However, the method entails a loss of porphyrin *a* which is variable but may reach 20 p.c. Furthermore, aqueous hydrochloric acid alters porphyrin *a*. Falk and Rimington (1952) observed that after treatment with strong aqueous hydrochloric acid, a part of the porphyrin *a* became far more readily extractable by dilute hydrochloric acid; they also observed differences in R III/IV and other absorption ratios between the various fractions which they attributed to an alteration of porphyrin *a*. Recently, Lemberg (1955) has shown that aqueous hydrochloric acid converts porphyrin *aα* partly and reversibly into porphyrin *aβ*, which differs from the former in its easier extractability from ether by dilute hydrochloric acid, or by phosphate buffer pH 6·5, as well as in its chromatographic behaviour. The absorption spectra of the two compounds are, however, indistinguishable, both qualitatively and quantitatively. The deviation of absorption found by Falk and Rimington was due to the presence in their porphyrin *a* of cryptoporphyrin *a* which accumulates in the 4–6 p.c. HCl fractions, as well as some yellow substance (see below) which lowers R III/IV. The spectrophotometric determination after HCl-extraction (methods A and B_1) can therefore be used. However, it gives values which may be about 10–20 p.c. too low, owing to the incomplete extraction of the porphyrin by 20 p.c. HCl.

In later experiments from A87 onwards, methods B_2 and B_3 of Table 1, it was found possible to decrease the formation of the yellow substance to such an extent that R III/IV was above 1·8, and in some instances about 2·0 after removal of the other porphyrins with 8 p.c. HCl. This allowed the estimation of porphyrin *a* without the need for extraction with strong hydrochloric acid from ether. Such solutions have been purified by chromatographic adsorption on cellulose powder to give spectroscopically pure porphyrin *aα* with R III/IV of 2·04 in a yield of over 75 p.c.; these experiments will be described in detail in a later paper (Lemberg and Stewart, 1955).

VARIATIONS OF METHOD.

Pre-extraction of the mince with saline. Since the removal of protoporphyrin in the method described above offers no difficulties, the washing of the mince with physiological saline to remove myoglobin was usually omitted. It was shown in one experiment summarized in Table 2 that pre-extractions with saline result in a large decrease of the yield of protoporphyrin, but only slightly affect that of porphyrin *a* or of cryptoporphyrin *a*.

TABLE 2.

Relative porphyrin yields from heart muscle with and without extraction with saline.

The values are in terms of "total extinction E" (D × vol. in ml./cm.) per kg. of fresh muscles.

	Not extracted	Saline extracted
Total haemins $E_{390 m\mu}$ in acetone HCl	41,600	6,750
Protoporphyrin E_{553} in 3 p.c. HCl	1,700	291
Cryptoporporphyrin E_{559} in 8 p.c. HCl	62	56
Porphyrin *a* E_{557} in ether	400	332

The saline washed muscle had only 16 p.c. of the protoporphyrin and a correspondingly low proportion of total haemin ($\epsilon_{390\ m\mu}$ measures predominantly protohaemin), but still contained 83 p.c. of its porphyrin *a* and 90 p.c. of its cryptoporphyrin *a*. The latter is therefore unlikely to have been formed from protohaemin, and we have found indeed no evidence for cryptoporphyrins being formed from haemoglobin under the conditions of our isolation of the porphyrins.

Thawing of frozen mince. Rapid thawing of frozen mince, suggested by Kiese and Kurz (1954), offered no distinct advantage over slow thawing at 4° C. Method B_3 gave 107±14 mg. per kg. of dry mince, R III/IV=1·81±0·02, slow thawing 102±7 mg. of R III/IV=1·89±0·07.

Water-content of the acetone-hydrochloric acid used for extraction. Preparations in which the haemins were extracted from mince dried by washing with acetone or alcohol have always resulted in poor yields of porphyrin *a* and low R III/IV. It was observed that the main difficulty arose when the porphyrin *a* was to be extracted from its ether solution by strong hydrochloric acid, when the lipoids retained a large proportion of the porphyrin in the ether. This was particularly noticeable in the procedure of Falk and Rimington. The yellowish substance remaining in the ether had a spectrum resembling that of porphyrin *a*, and a high Soret band in the ultra-violet, but ill-defined maxima and minima in the visible parts of the spectrum, a much higher absorption in the blue-green, and a low R III/IV. It probably consists of altered porphyrin *a* and some unsplit haemin. Attempts to extract porphyrin *a* with hydrochloric acid of more than 25 p.c. (w/v.) failed and the colour of such extracts was brown or rapidly changed from blue-green to brown; the porphyrin obtained from such solutions had a very low R III/IV (1·0–1·4).

The following experiment suggests that water-free acetone-HCl extracts a substance from heart muscle which causes the loss and alteration of porphyrin *a*. Three parallel experiments were carried out, (1) with method A as described above, (2) with precipitation of the homogenate of method A with neutral acetone, followed by the extraction of the acetone-dried precipitate with dry acetone-HCl, (3) as in (1) but adding to the main acetone-HCl extract of the wet homogenate, a dry acetone-HCl extract obtained from the supernatant of the homogenate by precipitation with neutral acetone. While method A yielded 19·7 mg. per kg. fresh muscle of porphyrin of R III/IV 1·92, in both other instances the yield was only 12·2 mg. and R III/IV 1·82.

It appeared possible that the above mentioned type of alteration during the extraction with strong HCl with formation of the yellow substance (pure porphyrin *a* is perfectly stable in conc. HCl for days) was due to the formation of peroxides formed from autoxidisable fatty acids. No increase of the yield was found when the extraction with acetone-HCl was carried out *in vacuo*, or under carbon monoxide, nor did the addition of α-tocopherol as an antioxidant have any effect; these experiments were, however, carried out with extraction by acetone-HCl containing 20 p.c. of water, so that the extraction of the destructive lipoid substance may already have been minimal.

The addition of small amounts of ferrous sulphate to the dilute hydrochloric acid used for transferring the haemins into ether was tried, since it appeared possible to destroy peroxides in this way. In one experiment with method A the yield was, indeed, increased from 11·4 mg. to 14·0 mg. per kg. of fresh muscle by the addition of ferrous sulphate at this stage. R III/IV, which was high without iron addition in this experiment (2·03 in the porphyrin extracted with 20 p.c. HCl, 1·93 in that extracted by 25 p.c. HCl), was further increased to 2·17 and 2·04 respectively.

Extraction of the haemins. Mixtures containing less than 0·7 p.c. (w/v.) of hydrochloric acid in 80 p.c. acetone-20 p.c. water proved less efficient, the same volume containing 0·45 p.c. HCl extracting only 77 p.c., and 0·3 p.c. HCl extracting only 34 p.c. of the amount extracted by 0·7 p.c. HCl. 3·7 l. of the 0·7 p.c. HCl mixture extracted from 1 kg. fresh muscle in the first extraction 27·0 mg., while the same volume extracted from the residue only 1·7 mg. of R III/IV=1·24, and none in the third extraction.

Pre-treatment of the wet mince with excess butanol at room temperature gave results similar to those obtained by its pre-extraction with neutral 80 p.c. acetone.

The omission of the dilution with water, raising the water content from 20 p.c. to 30 p.c., before freezing out fat at −15° C. caused the yield to drop from 89 to 57 mg. per kg. of dry weight, and the R III/IV from 1·88 to 1·74. For preparative purposes it is perhaps preferable to carry out the extraction with 80 p.c. acetone-HCl at −15° C. instead of at +4° C.; the yield was slightly lowered by this procedure, but less lipoid went into solution, and the later removal of fat by freezing could be omitted. This modification was not used in the experiments described above.

Precipitation of the haemins. The precipitation of the haemins from acid acetone solution by neutralization has been used by Negelein (1933) for the purification of haemin *a*. It appeared possible that the haemins would be sufficiently purified by this precipitation to make unnecessary their transfer into ether. This would offer distinct advantages for a large scale preparation. We carried out this neutralization by adjusting the pH to 6·9 with ammonia. As a step in our procedure of isolation, it proved, however, disadvantageous in two experiments, with yields of porphyrin *a* lowered from 96 and 109 mg. per kg. dry weight without haematin precipitation to 54 and 57 with precipitation, and R III/IV lowered from 1·93 and 1·81 to 1·51 and 1·68. In a third experiment the yield and R III/IV were the same as in a parallel experiment without precipitation. In some instances, the precipitation remained incomplete.

The precipitation also failed to prevent the lowering of R III/IV of the resulting porphyrin *a* which we had found (see above) when a crude acetone-HCl solution of the haemins was evaporated directly *in vacuo*. For this experiment, the precipitate was first dissolved in acetone containing a trace of hydrochloric acid; one half of this was brought into ether with dilute hydrochloric acid, the other half was evaporated directly. In the first half R III/IV of the porphyrins was 1·70, in the second only 1·40.

It has been mentioned above that part of the haemins crystallized from the ether solutions; this did not contain much haemin *a*. Attempts to precipitate the haemins from the ethereal mother liquor with light petroleum failed. Haemin *a* as well as protohaemin were present in

precipitate and filtrate, and although the filtrate appeared relatively faintly coloured, it contained predominantly haemin *a*, and too much of it to be neglected. A separate working up of precipitate and mother liquor gave the same results as the whole ether solution before separation. Pure haemin *a* is practically insoluble in light petroleum, but is kept in solution by dissolved lipoids.

The removal of the haem-iron. Iron can be reintroduced into porphyrin *a* (Lemberg and Stewart, 1955). If the reconstituted haemin *a* was again converted into porphyrin *a* by one of the methods described above, the porphyrin *a* had the same high R III/IV as before. When, however, a large excess of ferrous acetate was used in the ferrous acetate-HCl method, the resulting porphyrin *a* had a low R III/IV (1·44 instead of 1·99) and the yield was also decreased.

For a systematic study of the process small aliquots (about 1/20) of the crude haemin solution in ether from a preparation with 500 gm. fresh heart muscle were used. Ferrous acetate was replaced by sodium acetate+ferrous sulphate, varying the proportions of the reagents and the temperature. In these experiments, it was found that ferrous sulphate and hydrochloric acid could be added together without detriment, and later experiments showed that the addition of sodium acetate was unnecessary. A spectrophotometric method (Lemberg and Parker, 1955) was used which allows the estimation of porphyrin *a* in mixtures with proto- and cryptoporphyrin. In two experiments the values for porphyrin *a* thus determined were compared with the amounts determined in the usual way after extraction of the other porphyrins with 8 p.c. (w/v.) of hydrochloric acid; these are in good agreement (Table 3).

TABLE 3.

Conversion of haemins into porphyrins.

About 2 μ moles of haemins in 5 ml. glacial acetic acid +0·1 ml. saturated sodium acetate solution (abt. 200 μ moles), 80° C., splitting time 30 seconds.

μ moles Fe^{2+}	μ moles HCl	Porphyrin *a*	Proto-porphyrin	Crypto-porphyrin	Porphyrin *a* after extraction of other porphyrins with 8 p.c. HCl
36	3000[1]	0·59	0·71	0·04	0·60
36	3000[2]	0·59	0·69	0·03	0·61
18	3000[1]	0·55	0·68	0·10	—
18	1000[1]	0·56	0·78	0·04	—

[1] added after $FeSO_4$ [2] added together with $FeSO_4$

The results show that between 10 and 20 moles of Fe^{2+} per mole haemin suffice. Smaller amounts left some of the haemin unsplit. A final HCl concentration of about 1 p.c. suffices. The reaction is very rapid at 80° C., but requires about 5 minutes at 50° C., leaving some haemin unsplit.

Use of the Method for the Estimation of Porphyrin *a* in Small Amounts of Heart Muscle.

Method B_3 was used for these estimations. The results are given in Table 4. Except for the somewhat large yields in one experiment with 25 gm. of fresh ox heart and the somewhat low R III/IV with small amounts of muscle, the

TABLE 4.

Yields from different amounts of heart muscle.

Fresh ox heart mg.	Porphyrin *a* in mg. per kg. of fresh muscle	R III/IV
500	27·8, 33·2	1·83, 1·79
50	25·8, 29·5	1·93, 1·81
25	31·4, 39·2, 24·4, 26·2	1·62, 1·72, 1·88, 1·70
10	31·5, 27·3	1·54, 1·63
5	23·1, 27·2	1·82, 1·67

results agree well and show that the method can be applied with reasonable accuracy for very small amounts of heart muscle.

Porphyrin a *from hearts of other animals.* Pig and sheep hearts gave only slightly lower yields of porphyrin *a* than ox hearts. So far, these have been determined only with method A. While the average yield per kg. of fresh ox heart was 13·1±2·9 mg. of porphyrin *a*, sheep hearts gave 10·8 and 12·4 mg. and pig hearts 11·8 mg.

Other Methods of Isolation.

Method of Negelein (1933). In this method the haemins are extracted with acetone-hydrochloric acid from the wet mince. The haematins are then adsorbed from the neutralized solution on alumina (Wislicenus "Fasertonerde"). These adsorbates are exhaustively washed with a variety of solvents to remove lipoids, eluted with acetone containing some hydrochloric acid and reprecipitated by neutralization. These are lengthy procedures during which losses are unavoidable. A rough spectrophotometric analysis of the crude acetone hydrochloric acid extracts from the heart muscle still containing its myoglobin, based on the absorption at 640 and 390 mμ indicated that 1 kg. of fresh heart muscle contained about 220 mg. haemins, calculated as protohaemin which predominates. The amount isolated by Negelein in the stages mentioned above was only 32 mg. (by weight) per kg.; while the major part of the haemin lost is probably protohaemin, it can be shown (see below) that a great deal of haemin *a* must also have been lost.

The low iron content of the isolated haemin showed that one-third of it still consisted of iron-free impurities. Its haemochrome also showed protohaemochrome besides haemochrome *a* bands. An exact analysis of mixtures of haemin *a*, cryptohaemin *a* and protohaemin is still impracticable. A rough calculation of a mixture of protohaemin with haemin *a* is, however, possible, based on the following data of the specific extinction coefficients of pyridine protohaemochrome (ϵ_{pr}) and pyridine haemochrome *a* (ϵ_a) in 20 p.c. pyridine containing 1 p.c. NaOH:

$$\epsilon_{pr}^{558\ m\mu} = 46 \qquad \epsilon_{pr}^{587\ m\mu} = 2{\cdot}76$$

$$\epsilon_{a}^{558\ m\mu} = 14{\cdot}2 \qquad \epsilon_{a}^{587\ m\mu} = 33{\cdot}5$$

The two values for haemochrome *a* are based on estimations on our purest (amorphous) haemin *a*. These values give the equations:

$$\mu g.\ \text{protohaemin per ml.} = 22{\cdot}6 \times \epsilon_{558\ m\mu} - 9{\cdot}5 \times \epsilon_{587\ m\mu}$$

$$\mu g.\ \text{haemin } a \text{ per ml.} = 29 \times \epsilon_{587\ m\mu} - 1{\cdot}7 \times \epsilon_{558\ m\mu}$$

From this analysis, the fact that in the haemochrome of Negelein's crude haemin *a* the haemochrome bands at 587 and 588 mμ were of about equal strength, and the iron content, it can be calculated that Negelein's yield was only 14 mg. of haemin *a*[1].

Our own yields by this method were even smaller, varying between 9 and 11·6 mg. per kg. muscle of fresh heart.

The later stages of purification described by Negelein were not used since they involve large losses. Instead, it was attempted to remove the iron at this stage and to separate the porphyrins, as described above.

Great difficulties were, however, encountered in the removal of the iron from the haemins. After *in vacuo* evaporation of the acid acetone solution, the haemin was not entirely soluble in glacial acetic acid at room temperature. The remainder was brought in solution in a minimum of pyridine and then diluted with glacial acetic acid. This process can be used with advantage for the preparation of protoporphyrin from protohaemin, where the pyridine does not interfere with the removal of the iron if the final hydrochloric acid content is raised to about 3 p.c. Whether it is equally applicable to the haemin *a* has not yet been tested with pure haemin *a*, but it appeared preferable to lengthy heating in glacial acetic acid with or without ferrous acetate-HCl. The acetic acid and pyridine-acetic solutions were worked up separately. Both gave poor yields of porphyrin *a* and a considerable amount of porphyrins could not be brought into the ether phase with dilute sodium acetate-solution, but precipitated in the interface. A considerable amount of cryptoporphyrin was extracted by 8 p.c. hydrochloric acid. In contrast to the crude porphyrin *a* obtained by the method of Falk and Rimington (see below), the porphyrin *a* was quite stable even to concentrated hydrochloric acid. The yields were, however, very small, 1·0–2·5 mg. per kg. of fresh muscle, i.e., less than one-tenth of the yield obtained by our method. R III/IV varied between 1·56 and 1·65. It is evident that the method cannot be used for the measurement of the haem *a* content of heart muscle. Porphyrin *a* is partly converted into cryptoporphyrin *a* and partly into a modified porphyrin *a* of lowered R III/IV (about 1·4) which does not appear to be identical with cryptoporphyrin *a*.

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[1]Warburg and Gewitz (1951) observed that dithionite reduces haemochrome *a* to a haemochrome with protohaemochrome bands. Under the conditions chosen by us this reaction is, however, so slow that it can be neglected (cf. Lemberg and Stewart, 1955).

Method of Falk and Rimington (1952). In method A of these authors the haemins are extracted with acetone-hydrochloric acid from the dried mince, brought into ether, adsorbed on a column of MgO and washed on the column with ether; after dissolving the haemin-containing layer in glacial acetic acid, the haemins are brought back into ether and the iron is removed by the ferrous acetate-HCl method. Protoporphyrin is extracted from the ether solution of the porphyrins with 4 p.c. (w/v.) HCl. The porphyrin remaining in the ether had a low R III/IV of 1·20, which was partly due to the fact that crypto-porphyrin had not been removed. Extraction of the solid residue of evaporation with 25 p.c. (w/v.) HCl yielded 36–40 mg. of porphyrin with R III/IV 1·52 per kg. of dry mince.

Actually, a large amount of crytoporphyrin can be shown to be present if the ether solution after removal of the protoporphyrin is extracted with 8 p.c. (w/v.) HCl. The adsorption of the haemins on MgO fails to remove a lipoid substance which seriously interferes with the extraction of porphyrin *a* with hydrochloric acid. 25 p.c. HCl fails to extract substantial amounts of porphyrin *a* from the ether solution. The extraction of the crude solid porphyrin with 25 p.c. HCl at —10° C., according to Falk and Rimington, extracts only ¼–⅓ of the porphyrin present in the ether. Concentrated HCl extracts more, leaving still a large amount of yellow substance (see above) in the ether. Such extracts are unstable and their blue-green colour soon changes to brown. Even if the porphyrin is brought from the hydrochloric acid solutions immediately into fresh ether by dilution with water and neutralization with solid $NaHCO_3$, R III/IV is lowered to 1·4. Our total yield of porphyrin *a* per kg. of dry mince was only 18–20 mg., and an additional 14–18 mg. of cryptoporphyrin *a*. A comparison with the yields obtained by our method B_3 shows that almost ⅔ of the porphyrin were lost and of the remainder almost half was converted to crypto-porphyrin *a*. Even the remaining 1/6 was not unaltered as the low R III/IV 1·5–1·6 shows.

Method of Kiese and Kurz (1954). These authors have confirmed the finding of Lemberg (1953) that the optimal water content of the acetone-HCl extract lies between 20 and 30 p.c. The results of their method which isolates haemin *a* from protohaemin without conversion to the porphyrins will be compared with our results in the succeeding paper of Lemberg and Stewart (1955).

Porphyrin *a* from Cytochrome Oxidase Preparations.

Dannenberg and Kiese (1952) isolated a porphyrin *a* of R III/IV 1·6 and absorption bands at 645, 580, 555, and 515 mμ from a cytochrome oxidase preparation free from cytochromes *b* and *c*; in a short note Kiese (1952) reports that an unspecified alteration of the method of removal of iron yielded a slightly different porphyrin *a* with absorption bands at 646, 586, 560 and 518 mμ, with R III/IV 1·75. Our porphyrin *a* has a higher R III/IV 2·04, but absorption bands II, III and IV at somewhat shorter wavelengths (646, 581, 558 and 517 mμ).

The preparation of cytochrome oxidase and separation from the other cytochromes, in particular from cytochrome *b*, cannot be carried out quantitatively. We found, e.g., that the residue left after grinding heart muscle mince in 0·02 M phosphate buffer at pH 7·3 with sand according to Keilin and Hartree (1947) still contained a large part of the haemin *a*. Porphyrin *a* could be obtained from such a residue by our method A in a yield of 6–9 mg. per kg. of fresh muscle, i.e., 45–65 p.c. of the yield obtained from fresh heart muscle. This agrees well with the extraction of 42 p.c. of the oxidase found in enzymatic estimations by Keilin and Hartree. We have not tried extraction of the oxidase with buffers of higher alkalinity which, according to Haas (1943) and Dannenberg and Kiese (1951), but not according to Keilin and Hartree, extract more of the oxidase.

A preparation of the oxidase, according to Straub (1950), followed by extraction with acetone-hydrochloric acid and preparation of porphyrin, gave a very small yield of porphyrin of R III/IV 1·23 which appeared to consist mainly of cryptoporphyrin.

DISCUSSION.

Our results prove that porphyrin *a* is the prosthetic group of both cytochromes a_3 and *a;* apart from porphyrin *a* and protoporphyrin only cryptoporphyrin *a* is obtained from heart muscle, and in our methods the yield of cryptoporphyrin is so small that it cannot possibly account for either cytochrome a_3 or *a*. The amount of porphyrin *a* which can be isolated is in reasonably good agreement with the molar concentration which can be calculated for the sum of cytochromes *a* and a_3 in a rather indirect manner. Ball, Strittmatter and Cooper (1951) have arrived at a figure of 4·35 μ moles for cytochrome a_3 and of 17·4 μ moles for cytochromes *a* and a_3 in fresh heart muscle. These values are too low, since the differential extinction of CO-cytochrome oxidase and reduced cytochrome oxidase at 590 mμ was divided by the molar extinction coefficient of the CO-combined oxidase found in the photochemical absorption spectrum by Warburg, thus neglecting the absorption of reduced oxidase uncombined with CO at this wavelength. In a footnote in a later paper (Ball and Cooper, 1952), the error thus produced is estimated to be about 25 p.c. which would bring the values for cytochrome a_3 to 5·45 μ moles, and for cytochromes $a+a_3$ to 21·7 μ moles, with a ratio of cytochrome *a*/cytochrome a_3=3 and of cytochromes $a+a_3$/cytochrome *c* of about 1·5. The last ratio is based on the estimations of the cytochrome *c* content in ox heart muscle, 14·7 μ moles, by Junowicz-Kocholaty and Hogness (1939).

From the concentration of cytochrome *c*, those of cytochrome a_3+a can also be roughly calculated, using the ratio of cytochromes, $a_3:a:c=1:2:1{\cdot}6$, given by Chance (1952). These ratios differ from those of Ball; $(a_3+a)/c=1{\cdot}9$ instead

of 1·5 and $a/a_3=2$ instead of 3. Chance's ratios would correspond to 28 μ moles a_3+a per kg. of fresh heart muscle. These ratios are, however, calculated from the absorption ratios on the assumption that the molar extinction (reduced minus oxidized) of cytochrome *a* is the same as that of lactoperoxidase, and that of cytochrome a_3 the same as that of verdoperoxidase; both of these assumptions are unproven.

Assuming a molecular weight of 780 for porphyrin *a*, corresponding to the molecular weight found for crystalline haemin *a* (cytohaemin) of Warburg and Gewitz (1951) of 870 calculated from its iron content, our average yield with method B_3 corresponds to 37 μ moles. On the basis of the specific extinction of porphyrin *a* (cf. Lemberg and Stewart, 1955), a somewhat higher molecular weight of 845 is derived, which would correspond to a content of 33 μ moles.

This comparison shows that the amount of porphyrin isolated by our method somewhat exceeds that expected from measurements of the concentrations of cytochromes $a+a_3$, but in view of the very indirect calculations, by which the latter have been obtained, the agreement must be considered as satisfactory.

SUMMARY.

A method for the preparation in high yield of porphyrin *a* from ox heart muscle is described which allows its spectrophotometric determination after complete removal of other porphyrins, but without considerable losses of porphyrin *a*. This method gives spectroscopically pure porphyrin *a* in a yield of about 100 mg. per kg. of dry weight. Very little cryptoporphyrin *a* is formed as a by-product.

The porphyrin *a* thus prepared differs from preparations by other workers in its absorption spectrum, particularly in the high ratio of $\epsilon_{558\ m\mu}/\epsilon_{517\ m\mu}$ (R III/IV).

A comparison of the yields of porphyrin *a* with the cytochrome content of heart muscle and the absence of significant amounts of other porphyrins prove that porphyrin *a* is the iron-free prosthetic group of both cytochrome a_3 (cytochrome oxidase) and cytochrome *a*. The yield of porphyrin *a* is almost quantitative.

The method can be used for the estimation of cytochromes $a+a_3$ in very small amounts of heart muscle. Earlier methods are investigated and the factors which influence yield and purity of porphyrin *a* are discussed.

REFERENCES.

Ball, E. G., Strittmatter, C. F. and Cooper, O. (1951): J. biol. Chem., 193, p. 635.
Ball, E. G. and Cooper, O. (1952): *Ibid.*, 198, p. 629.
Barrett, J. and Lemberg, R. (1954): Nature, 173, p. 213.
Chance, B. (1952): *Ibid.*, 169, p. 215.
Dannenberg, H. and Kiese, M. (1952): Biochem. Z., 322, p. 395.
Falk, J. E. and Rimington, C. (1952): Biochem. J., 51, p. 36.
Granick, S. (1952): Fed. Proc., 11, p. 221.

Haas, E. (1943): J. biol. Chem., 148, p. 481.
Junowicz-Kocholaty, R. and Hogness, T. R. (1939): *Ibid.*, 129, p. 569.
Keilin, D. and Hartree, E. F. (1947): Biochem. J., 41, p. 500.
Kiese, M. (1952): Naturwiss., 39, p. 403.
Kiese, M. and Kurz, H. (1954): Biochem. Z., 325, p. 299.
Lemberg, R. (1953): Nature, 172, p. 619.
Lemberg, R. (1955): Annales Academiae Scientiarum Fennicae, Series A II, Chemica, N: 60.
Lemberg, R. and Falk, J. E. (1951): Biochem. J., 49, p. 674.
Lemberg, R. *et al.* (1949): Abstr. Commun. 1st Internat. Congress Biochem., p. 351.
Negelein, E. (1932): Biochem. Z., 248, p. 243.
Negelein, E. (1933): *Ibid.*, 266, p. 412.
Rawlinson, W. A. and Hale, J. H. (1949): Biochem. J., 45, p. 247.
Rawlinson, W. A. *et al.* (1949): Abstr. Commun. 1st Internat. Congress Biochem., p. 378.
Rimington, C. *et al.* (1949): *Ibid.*, p. 379.
Straub, F. B. (1950): J. biol. Chem., 183, p. 89.
Warburg, O. and Gewitz, H. S. (1951): Z. physiol. Chem., 288, p. 1.

PORPHYRINS WITH FORMYL GROUPS VI

PURIFICATION AND PROPERTIES OF PORPHYRIN *a*

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In the preceding paper of this series (Lemberg, Bloomfield, Caiger and Lockwood, 1955), we have described the isolation from heart muscle of porphyrin *a* in a form practically free from other coloured compounds and sufficiently pure for its quantitative estimation by spectrophotometry, but still containing some colourless lipoid impurity. The present paper continues this study. It deals with attempts to remove impurities and establishes the specific extinctions and other properties of pure porphyrin *a*.

During this study new properties of porphyrin *a* were discovered, in particular its conversion by hydrochloric acid into a mixture of two spectroscopically identical and interconvertible forms which we have called porphyrin *aα* and porphyrin *aβ*.

Three different procedures have been used for the further purification of porphyrin *a*.

(1) Fractional extractions from ether by hydrochloric acid and by phosphate buffer.

(2) Chromatography on columns of cellulose powder and of silica gel.

(3) Fractional extractions of the solid porphyrin with solvents after procedures 1 or 2.

Fractional extraction from ether by hydrochloric acid of different concentrations was the method adopted first. This was also applied in attempts to isolate porphyrin *a* from spectroscopically different alteration products which accompany it, if obtained by one of the earlier methods, our own as well as those of Negelein (1933) and Falk and Rimington (1952). After it had been established that the spectrum of even moderately pure porphyrin *a* is not altered by even strong hydrochloric acid, it appeared possible to remove contaminating lipoids by repeated passage between ether and hydrochloric acid, particularly since porphyrin *a* becomes successively more easily extracted by dilute hydro-

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chloric acid. This was at first believed to be due to the gradual removal of lipoid, but Lemberg (1955) has shown that it is in fact largely due to the gradual transformation of the original porphyrin ($a\alpha$) into a more strongly basic (also more strongly acidic) porphyrin ($a\beta$) in the aqueous hydrochloric acid phase. Further evidence for this conversion is described in the present paper.

The two porphyrins, $a\alpha$ and $a\beta$, do not differ in their absorption spectra; but they differ widely in their distribution between ether and dilute hydrochloric acid, in their distribution between ether and phosphate buffer, and in their chromatographic behaviour; and these differences are not due to different degrees of purity. They are interconvertible. Their complete separation is, however, difficult and much material is lost in the process. We expected that porphyrin $a\beta$, which readily passes from ether into 3–5 p.c. hydrochloric acid, while porphyrin $a\alpha$ requires about 15 p.c. HCl, would be more readily obtained pure, since it appeared unlikely that lipoid would pass into the dilute acid phase. Our experiments have, however, failed to justify this expectation, and have also shown that porphyrin $a\beta$ is somewhat less stable than porphyrin $a\alpha$, being more readily transformed into a chlorin.

The chromatographic procedures (2) yield practically only porphyrin $a\alpha$, which is partly converted into porphyrin $a\beta$ on standing in dilute hydrochloric acid. It would, nevertheless, be premature to consider porphyrin $a\beta$ as a mere artifact, since heating in acid solution converts it into porphyrin $a\alpha$; heating in acid solution is necessary for the removal of iron from the haemin. Thus it is possible that porphyrin $a\alpha$ is an artifact derived from porphyrin $a\beta$.

Even after the purification procedures (1) or (2) the porphyrin tenaciously retains some lipoid. Fractional extractions of the solid porphyrin with light petroleum ether, acetone, benzene and chloroform, gave fractions of different specific extinctions. The porphyrin, unless highly impure, is insoluble in light petroleum and becomes, with treatment, gradually less soluble in ether and acetone. When the specific extinction coefficient at 558 mμ (band III) in acetone solution (ϵ_{sp}^{558}) reached 19·5–19·9, further fractionation did not increase it. This value was reached with procedures (1)+(3) as well as (2)+(3), and both with porphyrin $a\alpha$ and with porphyrin $a\beta$. This value of 20 for ϵ_{sp}^{558} for our purest porphyrin *a* is the one we have used in the preceding paper (Lemberg, Bloomfield, Caiger and Lockwood, 1955), and in this paper is assumed to represent the extinction of pure porphyrin *a*.

We describe some of the properties of porphyrin *a*, particularly the absorption spectra in the visible and ultraviolet part of the spectrum. Some of the compounds of porphyrin *a* are also investigated. Porphyrin *a* is converted into haemin *a* and the latter reconverted into the porphyrin. This allows the comparison of the haemin *a*, reconstituted from the porphyrin *a* obtained by our method, with haemin *a* isolated directly without removal of iron by other workers (Warburg and Gewitz, 1951; Kiese and Kurz, 1954).

EXPERIMENTAL.

Spectrophotometry. Absorption spectra and extinction were measured with a Hilger Uvispek Photoelectric Spectrophotometer. Measurements were carried out in cells covered with a cover slip. In order to avoid errors by evaporation, particularly in ether solution, the following method was used. The first sample was used for locating the exact position of the maxima, and in some instances of the minima of absorption. In a second, fresh sample the quantitative measurement at the chosen wavelength (usually 558 mμ in ether or acetone) was carried out at once. Ratios of the extinctions at two different wavelengths were then determined by measuring the density at one wavelength between two measurements at the second wavelength; these latter two measurements did not differ by more than 5 p.c. even on a hot day, and usually by far less. This method was particularly used for the measurement of the ratios of band III and IV (R III/IV) in neutral ether solution, which ratio is a good criterion of freedom of porphyrin *a* from other porphyrins.

All the solvents were of A.R. quality. Acetone was dried over sodium sulphate and redistilled. Dioxane was purified by distilling over metallic sodium. Commercial "chemically pure" chloroform was found to contain some zinc and had to be redistilled.

Distribution between ether and aqueous phases. Usually the ether phase was extracted three times with the aqueous phase, using about 1/6 of the volume for each extraction. In most instances, the second extract contained far less porphyrin than the first, and the third was practically colourless. If it still contained porphyrin, two more extractions were carried out. All hydrochloric acid concentrations are given in percent (w/v.).

From an aqueous HCl phase porphyrin *a* was driven back into ether by gradual neutralization of the acid under a layer of ether in a large Erlenmeyer with solid sodium bicarbonate with frequent shaking, until the solution had become neutral to Congo red paper, avoiding a large excess of $NaHCO_3$; a slight excess of dilute phosphoric acid was then added at once and the porphyrin extracted with ether in a shaking funnel. The solution was a striking raspberry-red. From solutions in alkaline solutions or phosphate buffer porphyrin *a* was driven back into ether by acidification with dilute phosphoric acid, the opalescent green colloidal solution being at once shaken with ether in a shaking funnel. Repeated vigorous shaking with ether was necessary to complete the extraction. Almost colourless opalescent solutions can still yield much porphyrin *a*.

Distribution coefficients between HCl-saturated ether and ether-saturated aqueous hydrochloric acid solutions were measured at room temperature by determining spectrophotometrically the amount in the ether solution before and after extraction with an equal volume of the aqueous phase.

Countercurrent distribution. The "diamond" method of Bush and Densen (1948) with n=5 was used, with ether-saturated aqueous HCl of different concentration as lower phase, and ether saturated with the aqueous HCl as upper phase. This yields finally 10 fractions. The separation was carried out in glass-stoppered measuring cylinders, and the porphyrin was measured spectrophotometrically in the 5 ether phases directly, and in the 5 aqueous HCl phases after driving back quantitatively into ether by neutralization. The technique revealed distinct deviations from normal behaviour of mixtures, which later found its explanation in the partial interconversions of porphyrins *aα* and *aβ* in aqueous hydrochloric acid during the fractionation.

Chromatography. Porphyrin *a* (free acid) is readily adsorbed on alumina but cannot be easily eluted without alteration. It was observed that porphyrin *a* was partly adsorbed by filter paper when its solutions in ether were filtered. This led to the use of cellulose powder (Genuine Whatman Cellulose Powder, Standard Grade, Balston Ltd.).

The dry powder is tamped with a tampon in a column of 35 mm. diameter to a height of about 25 cm. and washed with a mixture of equal parts of ether and light petroleum (b.p. 60–80°). Such a column adsorbs the porphyrin *a* from 500 gm. of heart muscle from its solution in equal parts of ether and light petroleum. A brown-red ring forms on the top of the column. After thorough washing with ether plus light petroleum followed by small amounts of ether, ether elutes the greater part of the porphyrin; the column is then eluted with a mixture of acetone and ether, and finally with methanol.

Silica gel was prepared according to the method of Lucas and Orten (1951). It was freshly hydrated before use by mixing it carefully with the same weight of distilled water in a mortar, and the column prepared from an ether or light petroleum slurry. This silica gel does not adsorb porphyrin *aa* from an ether solution, but retains porphyrin *aβ* on the top of the column. Porphyrin *aβ* is partly eluted with ether containing 20 p.c. of acetone, completely by acetone containing a little methanol.

Fractional solution of porphyrin a *in organic solvents and measurement of specific extinction.* Ether solutions of the porphyrin are dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue is taken up in a small volume of dry acetone and this solution is evaporated in thin-walled weighing vessels (weight about 2 gm.) in a vacuum desiccator over dry silica gel and paraffin wax, keeping the desiccator in the dark. The solid brown-green porphyrin sticks to the walls of the vessel and impurities can be extracted by repeated applications of hot light petroleum (b.p. 60–80°). With the purest preparations, this may dislodge some of the porphyrin from the vessel walls in fine flakes; in this instance, the light petroleum is decanted cautiously, leaving most of the porphyrin in the vessel, while the remainder is collected on a small G3 porous glass filter in a thin tube. The weighing vessel containing the porphyrin is then dried again in a vacuum desiccator, closed and weighed on the microbalance.

The porphyrin *a* is then dissolved in a few ml. of hot acetone and quantitatively rinsed into a 10 ml. measuring flask or cylinder through a small funnel. The empty vessel is then again dried and weighed, while the acetone solution is used for the measurement of the extinction. In this way, the specific extinction of about 1 mg. of the porphyrin can be measured with an accuracy in which the error of weighing is smaller than that of the spectrophotometric measurement. Fractional solution of the porphyrin (see below) can be carried out in the weighing vessel itself, or if larger amounts are available, by filtration through sintered glass tubes.

In some earlier preparations, porphyrin *a* was precipitated from its chloroform solution with light petroleum. Such precipitates were microcrystalline under the polarization microscope, but it could not be excluded that the double refraction was due to colourless impurities. Chloroform solutions were, however, found to be less stable than acetone solutions, and in particular, evaporation of the chloroform on a hot water bath occasionally caused a change of colour from raspberry-red to green or brown, indicating an alteration of the porphyrin.

Purification of porphyrin a *by fractional extraction with aqueous hydrochloric acid of different concentrations.* The absorption spectrum of porphyrin *a* remains unaltered, with no decrease of total extinction, when it is extracted from ether by 20 or 30 p.c. HCl, allowed to stand in the acid solution for 40 hours in the dark at room temperature, and then driven back into ether by neutralization with $NaHCO_3$ as described above.

In the experiments summarized in Table 1 different preparations of crude porphyrin *a* were fractionally extracted with 5, 10, 15 and 20 p.c. HCl from the ether solution. The preparations with R III/IV below 1·8 were derived from preparations by the methods of Negelein (1933), of Falk and Rimington (1952), and by some of our own extracts in which acetone-dried heart muscle was extracted with dry acetone-HCl. The preparations with high R III/IV were prepared by the methods described by Lemberg, Bloomfield, Caiger and

Lockwood (1955) in the preceding paper, either by methods B_2 and B_3, directly after removal of proto- and cryptoporphyrin with 8 p.c. HCl, or by methods A and B_1, after extracting the porphyrin *a* from ether with 20 p.c. HCl and returning into ether by neutralization.

TABLE 1.

Increase of purity of porphyrin *a* by fractionation with hydrochloric acid.

R III/IV before fractionation	After fractionation		
	Purest HCl fraction(s) p.c. HCl	R III/IV of purest fraction(s)	p.c. of total
1·53	15	1·65	35
1·54*	10	1·90	31
	15	1·81	55
1·59	10	1·67	14
1·60*	10+15	1·81	76
1·61	15	1·80	51
1·61	15	1·80	57
1·62	15	1·81	6
1·65	15	1·76	30
1·68	15	1·78	29
1·69	10	1·83	19
1·72	15	1·77	21
1·73	—	1·73	—
1·77	10—15	1·94	73
1·77	10—15	1·86	53
1·77	15	1·88	39
1·78	10	1·82	37
1·78	15	1·97	50
1·79	5	1·87	54
1·81	10—15	1·98	54
1·81	10—20	1·90	61
1·82	15—20	1·92	76
1·87	10—15	1·98	66
1·87	—	1·84	—
1·87	—	1·88	—
1·89	15	1·98	76
1·90	—	1·96	—
1·90	15	1·99	46
1·93	—	1·95	—
1·94	10	2·06	32
1·94	5—10	2·09	44
1·98	15	2·15	41

* Contain traces of proto- and cryptoporphyrin.

Table 1 shows that in most experiments 35–75 p.c. of the total porphyrin could be obtained in a spectroscopically purer form, indicated by a higher R III/IV, but occasionally there was no purification. The fraction which contained the purest porphyrin *a* was usually the fraction extracted by 15 p.c. HCl. In two experiments marked with an asterisk, a small amount of cryptoporphyrin, which had escaped the preceding extraction with 8 p.c. HCl, was found in the fractions extracted by 5 and 10 p.c. HCl. Such fractions had a low R III/IV. In the other, less pure fractions, R III/IV was 1·5–1·6 or above, and no cryptoporphyrin was found to account for the low R III/IV. Some of the impurity was obtained in the form of a green, glittering skin at the interface between ether and 10 or 5 p.c. HCl. It appeared crystalline to the naked eye, but was found under the microscope to consist of amor-

phous green flakes. It was rapidly redissolved in ether if its suspension in ether was shaken with water which removed the hydrochloric acid. The porphyrin obtained in this way had a low R III/IV (1·25–1·45); occasionally it may occlude some pure porphyrin *a* when R III/IV is higher, and a repetition of the procedure will separate the low R III/IV porphyrin in the re-formed skin, while the pure porphyrin *a* remains in the ether. This impurity differs from cryptoporphyrin in its absorption spectrum, which, apart from the low R III/IV and a rather high R I/IV, resembles that of porphyrin *a*.

In no experiment was there any indication of change of R III/IV in the sum of all fractions, i.e., the absorption remained unaltered.

This process can therefore be used to recover porphyrin *a* from preparations which contain products of alteration. The total loss of extinction at 558 mμ is only about 10 p.c., but it gives pure porphyrin *a* only after much work and with considerable losses in the form of mixtures of low R III/IV; occasionally it does not work at all. It is therefore better to avoid the formation of the porphyrins of low R III/IV by the modification of the isolation technique described in methods B_2 or B_3 of the preceding paper, which yields immediately a porphyrin of high R III/IV.

Fractional extraction from ether with phosphate buffer preceding HCl fractionation. Porphyrin *a* is extracted from ether by M/5 secondary phosphate. The colour of the phosphate solution is green if dilute, red if concentrated. Ether solutions of the porphyrin were extracted fractionally with phosphate buffers of different pH. It was found that M/5 phosphate buffer of pH 6·5 extracted very little of a porphyrin having a low R III/IV, unless the porphyrin *a* had been taken previously into an aqueous hydrochloric acid phase (see below). Table 2 shows a number of experiments in which extraction with phosphate buffer of pH 6·5 preceded HCl fractionation.

TABLE 2.

Fractionation of porphyrin *a*, not previously extracted by aqueous HCl, with M/5 phosphate buffer of pH 6·5 and with hydrochloric acid.

R III/IV before	Percentages in each fraction Figures in brackets give R III/IV in each fraction						
	"+"[1]	"−5"	"−10"	"−15"	"−20"	"−25"	"skin"
1·63	10(1·51)	5(1·14)	12(1·66)	47(1·67)	25(1·67)	—	—
1·73	4(1·45)	2(1·75)	7(1·80)	23(1·85)	47(1·76)	13(1·55)	3(1·26)
1·78	4(1·56)	—	—	48(1·97)	26(1·84)	2(1·45)	20(1·66)
1·81	5(1·65)	—	10(1·90)	20(1·90)	61(1·90)	2(1·65)	2(1·15)
1·90	6(1·62)	—	—	40(1·96)	30(1·90)	—	23(1·74)
1·91	2(1·50)	—	8(2·00)	43(2·01)	43(1·99)	—	4(1·37)
1·98[2]	7(−)	1(−)	12(2·16)	44(2·08)	37(2·05)	—	—
2·02[2]	2(2·02)	—	4(1·70)	21(2·22)	45(2·09)	19(1·84)	9(1·86)

[1] The symbols "+" and "−" mean respectively extractability or non-extractability by phosphate buffer. Numbers refer to HCl concentration.
[2] After chromatographic purification (see below).

A comparison with Table 1 shows that this method purifies porphyrin *a* in all except the most impure preparation (R III/IV=1·63) more effectively than does HCl fractionation alone. The phosphate buffer extract and the "green skin" contained most of the impurities causing a low R III/IV. Most of the porphyrin *a* was extracted by 15 and 20 p.c. HCl, little by 5 and 10 p.c.

Repeated fractional extractions from ether with hydrochloric acid and phosphate buffer. An entirely different picture was obtained when porphyrin *a* which had previously been extracted from ether with 20 p.c. HCl, as in our methods A and B_1 of the preceding paper was investigated, or if the fraction obtained as described above, was refractionated. Gradually more and more porphyrin *a* accumulated in the fractions extracted from ether by phosphate buffer of pH 6·5 and by 5 p.c. hydrochloric acid. Table 3 shows the amount of porphyrin in per cent. of the total which was extracted by 5, 10, 15 and 20 p.c. HCl in the first fractionation without previous exposure to hydrochloric acid in the aqueous phase (line A), and the percentages extracted after prolonged fractionation (line C). Lines B and D will be discussed later.

TABLE 3.

Fractional extraction of porphyrin *a* from ether by hydrochloric acid before and after extraction from ether with aqueous hydrochloric acid.

	Percent of total extracted by hydrochloric acid of			
	5 p.c.	10 p.c.	15 p.c.	20 p.c.
A First fractionation	2	11	41	46
B First fractionation[1]	1	9	38	52
C Fourth fractionation	35	35	22	8
D B_1 after 16 hours' standing in 20 p.c. HCl	31	50	11	8

[1] After purification by chromatography.

While before HCl treatment little of the porphyrin was extracted from ether by 5 p.c. HCl and most of it was found in the 15 p.c. and 20 p.c. HCl fractions, after HCl treatment 5 p.c. HCl extracted about one-third of the porphyrin from ether and 15 p.c. HCl practically all. Similarly, the percentage of porphyrin *a* that could be extracted from ether by M/5 phosphate buffer of pH 6·5 had risen from 5 p.c. to an average of 30 p.c.

Repeated fractionations with hydrochloric acid and with phosphate buffer, the results of which are summarized in Table 4, were carried out in the following manner. After fractional extraction from ether with 5, 10, 15 and 20 p.c. HCl, each of the four fractions was brought back into ether, and the ethereal solutions extracted with M/5 phosphate buffer of pH 6·5, yielding eight fractions.

The symbol "+" signifies a fraction extracted by phosphate, and "−" a fraction left in the ether phase, and the numbers 5, 10, 15 and 20 indicate the hydrochloric acid which had extracted the fraction from ether; the eight fractions were thus "+5," "+10," "+15," "+20," "−5," "−10," "−15," "−20." Each of these eight fractions was again fractionally extracted with 5, 10, 15 and 20 p.c. HCl in a second fractionation. It was noted that most of the "+15" and "+20" fraction could now be extracted from ether by 5 p.c. HCl. The fractions now extracted by the same concentration of hydrochloric acid were united, independent of their previous HCl-extractability, but "+" and "−" fractions were kept separate (e.g., a 5 p.c. HCl fraction from a previous "+" fraction was united with those derived from "+10," "+15" and "+20" fractions, but not with those derived from the corresponding "−" fractions). Now, these eight fractions were subjected a second time to fractional extraction from ether by the phosphate buffer. While the "+5" and "+10" fractions yielded predominantly "+," the "+15" and "+20" fractions gave a considerable amount of "−," and all the "−" fractions

yielded some "+." The "++," "+−," "−+" and "−−" fractions were kept separate. These 16 fractions were subjected to a final (third) HCl fractionation. The fraction extracted by the same concentration of HCl was reunited as described for the second HCl fractionation. Table 4 gives the final result in these 16 fractions.

TABLE 4.

Distribution of porphyrin *a* between ether and hydrochloric acid and ether and phosphate buffer pH 6·5.

Fraction[1]	Percentage of total		R III/IV	R I/IV
++5	22·4		2·01—2·04	0·20
−+5	6·5	35·5	2·06—2·16	0·18
+−5	4·7		1·86—1·92	0·19
−−5	1·7		1·76	0·18
++10	4·2		1·87	0·26—0·33
−+10	3·1	35·5	1·97—2·03	0·20
+−10	9·8		1·95—2·03	0·17—0·19
−−10	18·4		1·97—2·01	0·17—0·19
++15	2·2		1·76—2·00	0·45—1·10
−+15	0·9	21·6	1·80	0·51
+−15	5·1		1·95	—
−−15	13·4		2·02—2·10	0·19—0·22
++20	1·0		1·68—1·73	0·38—0·60
−+20		7·5		
+−20	1·9		1·88—1·93	0·25—0·34
−−20	4·6		1·89—1·93	0·27—0·28

[1] Refers to fractionation history of fraction, e.g., −+5 means:—
Extracted by HCl of unstated strength (unstated because of various recombinations explained in the text), not extracted by phosphate buffer, extracted by HCl of unstated strength, extracted by phosphate buffer, and finally extracted by 5 p.c. HCl.

It can be seen that the major part of porphyrin *a* has accumulated in the "+5" and in the "−10" and "−15" fractions, and that these fractions do not differ in their absorption characteristics, of which R III/IV and R I/IV are given in the Table, cf. also Table 11 and Fig. 4. Most of the porphyrin is now extracted by 15 p.c. HCl, and about one-third by 5 p.c. HCl. The small "+10," "+15" and "+20" fractions contain an impurity which increases R I/IV and also shifts the position of the maximum of band I from 646 mμ towards the red. This was found to be due to the presence of a chlorin (see below). Some chlorin also appears to be present in the "−20" fraction. Of the "−" fractions only the small "−5" fraction had a low R III/IV, due to the presence of a little cryptoporphyrin *a*.

Table 5 shows the behaviour of individual fractions in the final fractional extraction with hydrochloric acid. The "++5" fraction is practically completely extracted by 5 p.c. HCl, and all the other "+" fractions are also extracted by 5 p.c. HCl to a large extent. The "−" fractions are little or not at all extracted by 5 p.c. HCl. Where on the second phosphate fractionation, a previous "−" fraction had become a "+" (phosphate buffer extractable) fraction, it now behaves in the HCl fractionation as a "+" rather than as a "−" fraction, and if a previous "+" fraction had become a "−" fraction in the second phosphate fractionation, it now behaves as a "−" fraction.

TABLE 5.

Behaviour of individual fractions on HCl refractionation.

Fraction	Percentage of total porphyrin *a* extracted by HCl of			
	5 p.c.	10 p.c.	15 p.c.	20 p.c.
++5	92	4	1	0
+−5	70	30	0	0
−−5	25	70	5	0
++10	80	14	6	0
−+10	50	26	24	0
−−10	31	60	9	0
++15	38	30	22	10
−+15	53	30	17	0
+−15	6	37	42	15
−−15	0	47	43	10
−+20	65	16	19	0
+−20	0	32	—	68
−−20	0	27	37	36

Table 6 shows the behaviour of individual fractions in the second phosphate fractionation. The "+5" and "+10" fractions have substantially remained "+," though giving some "−," while the "−" fractions and the "+15" fractions yielded predominantly "−" though giving some "+."

To sum up, the fractionations have separated porphyrin *a* into two spectroscopically indistinguishable fractions (see also Table 11 and Fig. 4), of which one is readily extracted from ether by 5 p.c. HCl as well as by phosphate buffer of pH 6·5 (porphyrin $a\beta$), whereas the other requires 10–15 p.c. HCl for extraction from ether and is only to a small extent extracted by phosphate buffer of pH 6·5 (porphyrin $a\alpha$). No complete separation has been achieved, however, except for the "++5" fraction which appeared to be practically uniform. There are some indications of an interconversion of the two porphyrins.

TABLE 6.

Behaviour of individual fractions on phosphate buffer refractionation.

Fraction	+5	+10	+15	−10	−15	−20
Percent extracted by phosphate buffer pH 6·5	84	64	29	31	17	29

Distribution coefficients between ether and hydrochloric acid. Attempts at determining the distribution coefficients of various fractions of porphyrin *a* between ether and hydrochloric acid were made before it was clearly recognized that aqueous hydrochloric acid modified porphyrin *a*. Approximate values could be obtained, since the amount of the porphyrin remaining in the ether phase was measured (see under "Methods"), and since this is little or not affected by the acid. Table 7 shows that different preparations of one and the same fraction gave similar, though still slightly variable results. The Table also shows the marked

TABLE 7.

Distribution coefficients (K HCl/ether) for different porphyrin *a* fractions.

Fraction	K
"+5"	(a) 3·08 p.c. HCl: 1·65 (b) 3·08 p.c. HCl: 1·06; 4·68 p.c. HCl: 3·22 (c) 2·78 p.c. HCl: 0·46; 3·54 p.c. HCl: 0·64; 4·68 p.c. HCl: 1·45
"<8"[1]	3·08 p.c. HCl: 0·50; 4·85 p.c. HCl: 1·53
"−10"	9·25 p.c. HCl: 0·144; 11·2 p.c. HCl: 0·59; 11·35 p.c. HCl: 1·51; 11·65 p.c. HCl: 13·0 reverse[2] 9·8 p.c. HCl: 0·29; 10·9 p.c. HCl: 1·22
"−15"	10·0 p.c. HCl: 0·6; 10·9 p.c. HCl: 1·0 9·9 p.c. HCl: 0·5; 10·9 p.c. HCl: 0·73
"−20"	15·05 p.c. HCl: 0·53; 15·5 p.c. HCl: 0·77; 15·95 p.c. HCl: 1·33; 18·25 p.c. HCl: 4·16 13·6 p.c. HCl: 0·22; 14·8 p.c. HCl: 18·9

[1] First three HCl fractions of countercurrent distribution of "−10" with 8 p.c. HCl.
[2] All extracted from ether by 20 p.c. HCl, brought back into ether by dilution.

effect of slight variations of hydrochloric acid concentration on the distribution coefficient, particularly in the more weakly-basic fractions. This is far greater than would be expected from the equations

$$K_a \cdot [HCl]_a^2 = K_b \cdot [HCl]_b^2$$

shown to hold for more strongly basic porphyrins by Granick and Bogorad (1953).

Attempts at separation by countercurrent distribution. Porphyrin *aβ* (the "++5" fraction) was subjected to the countercurrent distribution, as described under "Methods," between ether and ether-saturated 5 p.c. HCl (HCl-content after ether saturation 4·7 p.c.). The first three of the five HCl fractions contained 85 p.c. of the porphyrin and the last three of the five ether fractions 7·5 p.c. The porphyrin from the first three fractions had the absorption spectrum of pure porphyrin *a* ($\epsilon_{644}:\epsilon_{588}:\epsilon_{550}:\epsilon_{517}=0{\cdot}17:1{\cdot}18:2{\cdot}05:1$), while that of the last three fractions had a high R I/IV and a low R III/IV ($\epsilon_{634}:\epsilon_{588}:\epsilon_{550}:\epsilon_{517}=0{\cdot}33:1{\cdot}19:1{\cdot}76:1$). This is due to the presence of a chlorin which when isolated has a strong, sharp band at 656 mμ and three weaker bands in the green. Since R III/IV of the porphyrin before the countercurrent distribution had been 2·09 and R I/IV 0·18, the chlorin had been formed during the process. The relative amounts in fractions 1 and 2 should, according to the theory (Bush and Densen, 1948), be in the ratio of 1:5(1−p), and the ratio of the fractions 2 and 3 should be 1:3(1−p) where p is the fraction of the total extracted by an equal volume of hydrochloric acid, and K HCl/ether=p/1−p. From the amount found in fractions 1 and 2 (7·65 and 2·37 respectively), a K of 15·1 is calculated, while from those in fractions 2 and 3, K=7·35. The same kind of divergency was found throughout the experiments.

When the "−10" was subjected to countercurrent distribution in 8 p.c. ether-saturated HCl, 55 p.c. of the porphyrin was found in the first three fractions (HCl-phase), with K=17·4 calculated for fractions 1 and 2, and 5·4 from fractions 2 and 3. The last three fractions (ether phase) contained 35 p.c. of the porphyrin with a K of 0·065 calculated from fractions 10 and 9, and 0·20 from fractions 9 and 8. A comparison with the total distribution coefficient of the "−10" fraction before countercurrent distribution (see Table 7) shows that there must have been an alteration with the formation of a more strongly basic

porphyrin. The porphyrin extracted in the first three fractions (cf. footnote 1 to Table 7) had now a distribution coefficient similar to that of the "+5" fraction.

The "−15" fraction, in a countercurrent distribution with 10·85 p.c. HCl, gave 50 p.c. porphyrin in fractions 1–3 with K=14·9 calculated from fractions 1 and 2, and K=4·9 from fractions 2 and 3; and 31 p.c. porphyrin in the last three fractions with K=0·062 calculated from fractions 10 and 9. In another experiment with 13·5 p.c. HCl, 55 p.c. was in fractions 1–3, with K=11·5 calculated from fractions 1–2, and K=3·8 from fractions 2 and 3; the fractions 8–10 contained 31 p.c. with a K of 0·056 calculated from fractions 10 and 9.

The "−20" fraction, in a countercurrent distribution with 15·65 p.c. HCl, gave 26 p.c. porphyrin in fractions 1–3, with K=5·9 calculated from fractions 1 and 2, and K=3·0 from fractions 2 and 3; and 55 p.c. porphyrin in fractions 8–10, with K=0·07 calculated from fractions 10 and 9, and K=0·135 from fractions 9 and 8.

These experiments supported the assumption that there were mainly two different porphyrins present, *aα* and *aβ*, and that one was transformed into the other during the HCl extraction, particularly the more weakly basic porphyrin *aα* into the more strongly basic one, *aβ*. The various fractions were now studied by chromatographic methods.

Separation of porphyrins aα and aβ by chromatography. When a "−15" fraction was adsorbed on cellulose powder from a solution in equal volumes of ether and light petroleum, the greater part of the porphyrins could be eluted by ether from the column. It could not be adsorbed from its solution in ether on a column of silica gel (see "Chromatography" under "Methods"). We call this porphyrin *aα*.

In contrast, little of the porphyrin from a "+5" fraction was eluted from a cellulose powder by ether. The major part remained adsorbed on the top of the column; most of this was eluted by ether containing 20 p.c. of acetone, the last part by acetone containing 10 p.c. methanol, combining both fractions. The main part of the porphyrin from the "+5" fraction was adsorbed from its solution in ether on the top of a silica gel column; it could not be eluted by ether, but most was eluted by ether containing 20 p.c. acetone and the remainder by acetone containing some methanol. The two last fractions were combined. We call this porphyrin *aβ*. Ether containing small amounts of methanol or acetone can elute some of the porphyrin *aβ* from a silica gel column. If silica gel chromatography is to follow cellulose powder chromatography, it is necessary to remove methanol and acetone completely before the silica gel chromatography. This is best done by evaporating the eluates from the cellulose powder chromatography to dryness *in vacuo*, dissolving the residue in M/5 secondary phosphate and extracting with fresh ether after acidification with dilute phosphoric acid.

A solution of porphyrin *a* obtained without exposure to aqueous hydrochloric acid (method B_3 of the preceding paper) behaved like porphyrin *aα* in cellulose powder and silica gel chromatography (see below).

The total recovery in the cellulose powder chromatography was about 80 p.c., that in the silica gel chromatography 85–95 p.c. The colourless ether-light petroleum eluate from the cellulose powder columns contained lipoids, forming about 40 p.c. of the weight of the crude porphyrin in "+5" fraction and 50–70 p.c. in the "−" fractions. The absorption spectra of the two porphyrins *aα* and *aβ*, were not significantly different (see below).

Table 8 shows that a little porphyrin *aα* was present in the "+5" fraction, and a little porphyrin *aβ* in the "−" fractions. Silica gel separates these more effectively than cellulose powder, but the best results are obtained by cellulose powder chromatography which removes lipoid, followed by silica gel chromatography.

The figures shown for the per cent. of porphyrin eluted by ether from the cellulose column in the experiment with the "+5" fraction (line 1 of Table 1) are higher than the actual *aα*

TABLE 8.

Chromatography of porphyrin *a* fractions.[1]

Fraction	Eluted by ether from cellulose	Eluted by ether from silica gel	Fraction eluted by ether from cellulose, eluted by ether from silica	Fraction not eluted by ether from cellulose, eluted by ether from silica
"+5"	0, 31, 39	20, 23	34	7
"−10"	54, 60	83, 91	80	—
"−15"	47, 66	79, 84	93	75
"−20"	69, 87	78, 98	99·5	74

[1] The figures of columns 2 and 3 are percentages of the fractions of column 1. The figures of columns 4 and 5 are percentages of the chromatography fraction described in the heading of these columns.

content, since the silica gel chromatography shows that these ether eluates still contained more *aβ* than *aα*. Even the ether eluates from silica gel still contained some *aβ*, as well as some *aα*, since phosphate buffer of pH 6·5 extracted only 30–70 p.c. of the porphyrin from the ether eluates, but 90 p.c. from the adsorbate. Some *aα* has thus been removed in these eluates.

Similarly, the figures shown for the per cent. of porphyrin eluted by ether from the cellulose column in the experiments with the "—" fractions are lower than their actual *aα* content. The part adsorbed on cellulose powder also consists of a great deal of *aα*, as the silica gel chromatography of this fraction shows. Phosphate buffer of pH 6·5 extracted 30–70 p.c. from the adsorbates on silica gel, showing that these fractions contained some porphyrin *aβ*, but also some *aα*. Some *aβ* has thus been removed in the absorbate.

Purification of porphyrin a *not previously exposed to hydrochloric acid in the aqueous phase by chromatography.* Porphyrin *a* prepared according to method B_2 (without extraction with 20 p.c. HCl from ether) and B_3 of the preceding paper (Lemberg, Bloomfield, Caiger and Lockwood, 1955) was subjected to the chromatography on cellulose powder and silica gel columns as described above. In some instances some of the porphyrins from its crude solution in ether-light petroleum passed the cellulose column on washing with the ether-light petroleum mixture; these eluates were re-adsorbed on a second cellulose powder column. On the average, 80 p.c. of total porphyrin *a* was recovered, and 90 p.c. of this in the ether eluate. R III/IV of the porphyrin before chromatography was 1·67–2·07; with an average of 1·86. In the ether eluates from the column R III/IV varied between 1·92 and 2·13, with an average of 2·02. In the fractions extracted by 20 p.c. acetone-80 p.c. ether, which formed about 7 p.c. of the total, R III/IV was lower (1·54–1·99), and in the very small final methanol eluates it was very low (1·0–1·57). The latter thus consisted mostly of porphyrins other than porphyrin *a*.

A second chromatography on cellulose powder was followed by a chromatograph on silica gel. This gave practically pure porphyrin *aα* of which 96–99 p.c. was not adsorbed by the silica gel column, R III/IV being 2·00–2·16.

Re-chromatographing of the acetone-ether and methanol eluates after removal of acetone or methanol, showed that practically no porphyrin *aβ* was present, about 90 p.c. of these fractions now behaving also like porphyrin *aα*.

Interconversions of porphyrins a*α and* a*β.*

Porphyrin *aα*, purified by chromatography as described above, is extracted to a very small extent by phosphate buffer of pH 6·5 (see the last two lines of

Table 2). If, however, this porphyrin is first fractionally extracted with 5, 10, 15 and 20 p.c. HCl, and these fractions, after driving the porphyrin back into ether, are refractionated with phosphate buffer of pH 6·5, a total percentage of 36 p.c. is now extracted by this buffer. This is similar to the formation of 31 p.c. porphyrin extractable by 5 p.c. hydrochloric acid from ether after repeated fractionations with hydrochloric acid (see Table 3, lines B and D). Table 9 shows that the maximum percentage of porphyrin *aβ* ("+") is formed from *aa* in the 15 p.c. HCl fractions. At first glance this appears to contradict

TABLE 9.

Conversion of porphyrin *aa* to porphyrin *aβ* during the fractional extraction by hydrochloric acid from ether.

Experiments in duplicate.

Fractionation with phosphate buffer pH 6·5 preceding HCl-fractionation.							Fractionation with phosphate buffer pH 6·5 following HCl-fractionation.					
p.c. HCl	p.c. of total				p.c. "+" in fraction		p.c. of total				p.c. "+" in fraction	
	+		−				+		−			
5	0·4	0·6	0·3	1·4	57	30	0·3	0·2	0·4	0·9	43	18
10	0·6	0·6	3·6	10·5	14	5	1·6	2·1	3·8	5·8	30	27
15	0·9	2·5	39·2	44·4	2	5	15·9	23·8	24·8	24·4	39	49
20	1·7	3·3	53·3	36·7	3	9	9·2	9·7	44·0	33·3	17	23
	3·6	7·0	96·4	93·0			27	36	73	64		

our previous findings that *aβ* is completely extracted by 5 p.c. HCl from ether as well as by phosphate buffer of pH 6·5. It must be remembered, however, that the formation of porphyrin *aβ* from *aa* occurs in the aqueous acid phase, and that the amount of conversion depends on the concentration of the acid, which influences the rate of conversion, as well as on uncontrolled factors such as the length of time between extraction with acid and return to the ether. If the collected "+" fractions are refractionated with hydrochloric acid, indeed practically all is now extracted by 5 p.c. HCl.

The rates of reaction and the position of the equilibrium have not yet been studied in detail, but some exploratory experiments have been carried out. The figures in Table 10 give the percentage of porphyrin *a* which was extracted from ether by phosphate buffer of pH 6·5, after solutions of porphyrin *aa* and *aβ* had been standing in hydrochloric acid of different strength at room temperature for 16 hours, or had been heated at 90° C. for 20 minutes, and had been returned to ether by neutralization with sodium bicarbonate. Since a small percentage of porphyrin *aa* can be extracted by this buffer, the figures are slightly larger than the true porphyrin *aβ* content of the mixture. For the experiments in 5

TABLE 10.

Interconversion of porphyrin *aa* and porphyrin *aβ* in hydrochloric acid.

	HCl p.c.	p.c. +	
aa	30	9	16 hours at room temperature
	25	29	
	20	20, 29, 31, 33, 45	
	10	55	
	5	49	
aβ	20	61	extraction followed by driving back into ether in 5 mins.
	20	82	
aa	20	5	20 min., at 90° C.
aβ	20	6	

and 10 p.c. HCl, porphyrin *aa* had to be extracted first from ether by 20 p.c. HCl which was then diluted. The figures show that standing in aqueous hydrochloric acid converted some of the porphyrin *aa* into *aβ*, the maximum after 16 hours standing (about 50 p.c.) being in 10 p.c. HCl. Porphyrin *aβ* was also changed into *aa*, to the extent of 39 p.c., under conditions which changed *aa* into *aβ* to 45 p.c. maximally, so that full equilibrium was not reached. These findings explain the difficulties encountered in trying to separate *aa* and *aβ* completely by HCl-fractionation, as well as by counter-current distribution experiments.

It was surprising that heating in 20 p.c. HCl left porphyrin *aa* unaltered and converted porphyrin *aβ* completely into porphyrin *aa*. The porphyrin *aa* obtained in this way distributed in the same way as original porphyrin *aa* between 5, 10, 15 and 20 p.c. in the HCl fractionation, and could be partly reconverted into *aβ* by allowing the heated hydrochloric acid solution to stand at room temperature.

It was of interest to see whether porphyrin *aβ* would also be converted to porphyrin *aa* under the conditions used in the removal of iron from the haemins (1 minute heating at 85° C. in acetic acid containing 1 p.c. HCl). Porphyrin *aβ* treated in this way was converted, indeed, into porphyrin *aa*. Only 6 p.c. could be extracted by phosphate buffer of pH 6·5 from ether. If the haem or haems of the cytochromes *a* and a_3 were the iron complex of porphyrin *aβ*, porphyrin *aa* would still probably be produced from it during the removal of iron.

This very specific behaviour of porphyrin *a* might be useful for its isolation were it not for the fact that a part of the porphyrin is converted into ether-insoluble by-products during the heating of porphyrin *aβ* (loss 18 p.c.), and that some decrease of R III/IV also indicates that side reactions occur. Porphyrins *aa* and *aβ* are not converted into one another by sodium hydroxide (0·1 N as well as 2 N) at room temperature, or even at 90° C. in 50 minutes. The

stronger alkali, particularly at 90° C., causes, however, a considerable destruction, particularly of porphyrin *aβ*.

The addition of some iodine to the ether solutions of the two porphyrins also failed to cause any conversion.

Absorption spectra.

The absorption curve of an ether solution of porphyrin *a* in the visible part of the spectrum is shown in Fig. 1. The spectra of 12 samples of porphyrin *aa* and 5 samples of porphyrin *aβ* were not different either in the position of maxima and minima, or in the height of their extinction, except that bands II and III of porphyrin *aβ* appear to be slightly higher than those of *aa* (Table 11).

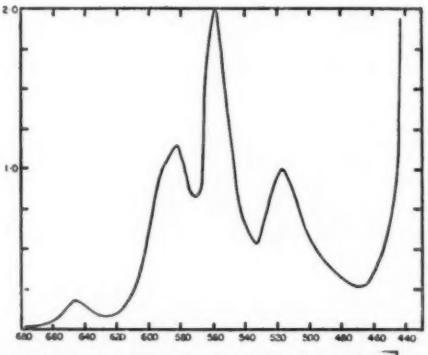

Fig. 1. Absorption spectrum of porphyrin *a* in ether.

The presence of impurities in porphyrin *a* preparations is revealed (a) by a lowering of R III/IV, (b) by an increase of R I/IV, and (c) by an increase of R II/IV and R II/III. The first phenomenon can be caused by the presence of some proto- and cryptoporphyrin, but cannot always be thus explained (see Lemberg, Stewart and Bloomfield, 1955, and Lemberg and Parker, 1955). Accompanying a low R III/IV, the positions of the two absorption bands III and IV are often shifted 1–2 mμ towards the blue. Such "low R III/IV" porphyrins have, for example, been obtained when a great excess of ferrous acetate is used for the removal of iron from haemin *a*, and they have formed the major part of the preparations by other authors and by our own earlier ones. An increase of R I/IV is caused by the presence of a chlorin which is more readily formed from porphyrin *aβ* than from *aa* and which can be removed by fractional extraction from ether with hydrochloric acid (see above). Parallel with the increase of R I/IV, the position of band I is shifted towards the red. An increase of R II/IV or R II/III is caused by minute traces of zinc or copper which convert part of the porphyrin to a metal complex.

TABLE 11.

Absorption spectra of porphyrin *a*.

Porphyrin *aa* in ether	Maxima	I[1] 647·1±0·9	II[2] 583·0	III[1] 558·8±0·5	IV[1] 517·0±0·6	V 412 mμ
	Ratios to $\epsilon_{517m\mu}$	0·176±0·017	1·12±0·04	2·00[3]±0·05	1·0	19·0
	Minima	625	573	534	470	
	Ratios to $\epsilon_{517m\mu}$	0·097	0·80	0·55	0·275	
Porphyrin *aβ* in ether	Maxima	I[1] 646·4±1·7	II[2] 583·0	III[1] 558·9±0·5	IV[1] 516·9±0·7	V 412
	Ratios to $\epsilon_{517m\mu}$	0·177±0·011	1·19±0·02	2·08[3]±0·06	1·0	18·1
	Minima	625	573	533	470	
	Ratios to $\epsilon_{517m\mu}$	0·077	0·84	0·52	0·255	
Porphyrin *a* in chloroform	Maxima	I 646	II 584·5	III 563·5	IV 520	V 418·5
	Ratios to $\epsilon_{520m\mu}$	0·26	1·40	2·11	1	18
	Minima	625	575	538	475	
	Ratios to $\epsilon_{520m\mu}$	0·145	1·30	0·66	0·34	

[1] Measured in the Hartridge Reversion Spectroscope. All other measurements in the Uvispek Spectrophotometer.

[2] Measured in the Spectrophotometer. In the Reversion Spectroscope the faint shoulder of this band (see Fig. 4) appears as a separate band at 595 mμ.

[3] In the purest *aa* and *aβ* fractions after chromatographic separation on silica gel a somewhat higher R III/IV was found: 2·09±0·09 for porphyrin *aa* and 2·15±0·08 for porphyrin *aβ*.

In acetone, the positions of the maxima and heights of their extinction were the same as in ether solution. In chloroform, the positions of the maxima were shifted towards the red end of the spectrum, and bands I and II were relatively higher (see Table 11), with a less marked minimum between bands II and III. The maximal extinction of band III in chloroform was the same as that in ether. The absorption spectrum in glacial acetic acid resembled that in chloroform (I, 643; II, 585; III, 561; IV, 520 mμ; ratio 0·28:1·65:2·26:1); the maximal extinction at band III was found to be somewhat variable, between 0·80 and 1·15 (average 0·96) times that in ether.

The solution of porphyrin a_α in 0·1 N sodium hydroxide appeared somewhat brownish-green as compared with the pure green of the solution of $a\beta$, but the absorption spectra revealed no distinct difference; both had two rather indistinct bands at 608 mμ and 570 mμ, with a faint shadow at 528 mμ. The absorption spectrum of a_α in M/5 secondary phosphate showed a maximum of the rather flat absorption curve at 573 mμ, a minimum at about 540 mμ, and a weak maximum at 528 mμ. Porphyrin $a\beta$ in phosphate buffer of pH 6·5 had a similar absorption spectrum. The Soret band was at 401 mμ.

The positions of the maxima, as well as the ratio of the bands of a solution of porphyrin *a* in hydrochloric acid vary somewhat with the concentration of the acid. Solutions of porphyrins a_α and $a\beta$ in 10 p.c. HCl were found to be indistinguishable (Table 12). The extinction at the maximum at 563·5 mμ in 10 p.c. HCl was only 0·72 that of the maximum at 558 mμ in neutral ether.

TABLE 12.

Absorption spectra of porphyrin *a* in aqueous hydrochloric acid.

p.c. hydrochloric acid		Maxima				Ratio I/II
		I	II[1]		III	
5		614·8	561·2 (centre	559·1)	527 (weak)	—
10	$a_\alpha+a\beta$	615·7	563·2	561·0	527	0·81
	a_α	615·0	563·6	561·5	—	—
	$a\beta$	615·5	563·5	561·5	—	—
15		616·9	565·1	563·1	528	0·85
20		618·6	565·5	563·6	528·5	0·88
25		619·0	564·8	564·2	—	0·90

[1] This band is unsymmetrical.

Fig. 3 shows the absorption curves of porphyrin *a*, protoporphyrin, chlorocruoroporphyrin dimethylester and monoformyldeuteroporphyrin dimethylester in dioxane. The porphyrin *a* was a preparation containing a mixture of porphyrin a_α and $a\beta$ of $\epsilon_{sp}^{558} = 18·1$ and R III/IV=1·91. The protoporphyrin was the crystalline free acid. The chlorocruoroporphyrin dimethylester was prepared from protoporphyrin dimethylester according to Lemberg and Parker (1952);

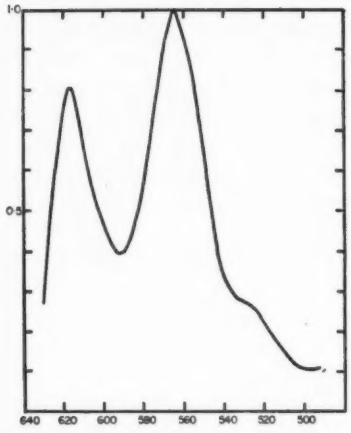

Fig. 2. Absorption spectrum of porphyrin *a* in 10 p.c. HCl.

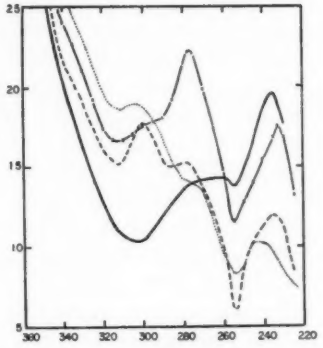

Fig. 3. Absorption spectra of porphyrins in the ultraviolet in dioxane.
Protoporphyrin (free acid) ———.
Chlorocruoroporphyrin (dimethyl ester) – – – –.
Monoformyldeuteroporphyrin (dimethyl ester) .....
Porphyrin *a* (free acid) x–x–x–x.

the monoformyldeuteroporphyrin dimethylester was the pure 4-formyl ester of m.p. 272–3° prepared by separation of the mixture of 2-formyl and 4-formyl esters (Lemberg and Parker, unpublished). The values for ϵ_{mM} are based on a millimolar extinction of 16·9 at 558 mμ for porphyrin *a*, of 14·8 at 504 mμ for protoporphyrin (Stern and Wenderlein, 1954), of 12·4 at 553 mμ for chlorocruoroporphyrin ester (calculated from the value of Stern and Molvig, 1936), of 16·0 at 551 mμ for 4-monoformyldeuteroporphyrin ester (Lemberg and Parker, unpublished).

The left side of the curve shows the fall of the Soret band. The other bands in the ultraviolet are far weaker, of the order of the bands in the visible part of the spectrum. The three formylporphyrins have a band at about 300 mμ which is probably characteristic of the formyl group, since protoporphyrin and its ester have a minimum of absorption in this region. The spectrum of porphyrin *a* shows this band only as a shoulder of a stronger band at 277 mμ. A weaker band in the latter region is also found in the spectra of the other porphyrins, that of protoporphyrin appearing as a shoulder or a band with maximum at 260 mμ. Since the specimen of porphyrin *a* used was only of about 90 p.c. purity, the relatively high 277 mμ band is possibly due to an impurity. All the porphyrins show a third band at about 235 mμ. This is very weak and slightly shifted to 243 mμ in monoformyldeuteroporphyrin which lacks a vinyl, somewhat stronger in chlorocruoroporphyrin which has one vinyl group, and strongest in protoporphyrin which has two vinyl groups. This band in porphyrin

a is almost as strong as that of protoporphyrin, indicating the presence of at least one vinyl group (or unsaturated group with the double bond conjugated to the pyrrole ring) as side chain.

The absorption spectra of porphyrin *aa* ("—20") and porphyrin *aβ* ("+4") were compared in methanol solution (Fig. 4). Methanol has the advantage over dioxane that it is more readily purified to a solvent without significant absorption in the 210–230 mμ region; it readily dissolves porphyrin *a* but could not be used in the experiments above, since it is a poor solvent for the other porphyrins. The estimations in Fig. 4 are relative to $\epsilon_{350\ m\mu}=1$, which was chosen for

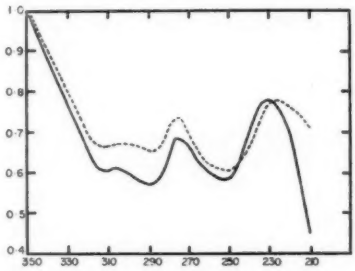

Fig. 4. Absorption spectra of porphyrin *aa* and *aβ* in the ultraviolet in methanol (relative to $\epsilon_{350\ m\mu}$).
Porphyrin *aa* ————.
Porphyrin *aβ* – – – –.

technical reasons; absorption at this wavelength is still dependent on the Soret band, and can be measured in the same solution and with the hydrogen arc lamp used for the measurements at the other wavelengths in the ultraviolet. The ultraviolet absorption spectra of the preparations of porphyrin *aa* and *aβ* measured differ slightly, particularly in the region of 230 mμ, while otherwise they are very similar. According to their specific extinctions at 558 mμ (15·0 and 14·2 respectively), the purity of the specimen of porphyrin *aa* was only 75 p.c., that of *aβ* 71 p.c. pure; the differences may therefore be caused by the nature of the impurities.

Further purification and measurement of specific extinction.

Neither the ether solutions obtained by the chromatographic method, nor those obtained by fractional extraction from ether with hydrochloric acid and with phosphate buffer, were free from colourless lipoid. This lipoid was removed by washing the solid with hot light petroleum, followed by fractional extraction by ether and acetone, as described above.

Before this purification ϵ_{sp}^{558} in acetone varied between 11·7 and 12·8 for *aa* obtained by the chromatographic method, between 13·2 and 18·4 for *aa*

obtained by the fractional HCl-extraction method ("—10," "—15" or "—20"), between 13·2–15·6 for $a\beta$ obtained by this method, and between 14·6 and 17·2 for porphyrin $a\beta$ further purified by chromatography. Table 13 lists the specific extinctions of a variety of such preparations. Further purification by solvent fractionation led in all instances to a product of ϵ_{sp} 18·8–19·9, and further fractionations failed to increase this specific extinction. The yield of this fraction varied with the initial degree of purity. While in one "—10" preparation, the yield was 91 p.c., and in one $a\beta$ preparation 70 p.c., it was far lower (10–40 p.c.) in other preparations.

TABLE 13.

Specific extinctions of porphyrin a in acetone, E, 0·1 p.c., 1 cm (ϵ_{sp}).

	$\epsilon_{sp}^{558\ m\mu}$(III)	$\epsilon_{sp}^{558\ m\mu} + \epsilon_{sp}^{517\ m\mu}$(III+IV)
$a\alpha$ (chromatography)	19·85	30·0
	17·2	25·5
	16·1	24·4
"—20"	19·9	29·6
	19·5	29·3
	17·2	25·6
	16·7	24·8
"—15"	19·5	28·8
	18·4	27·4
	17·0	25·1
"—10"	18·8	28·2
	18·3	27·4
	17·3	26·1
"+5"	19·5	29·5
	16·2	24·3
$a\beta$ ("+5" chromatographed)	18·9	27·8
	17·3	25·1
	15·9	23·7

TABLE 14.

Method of preparation[1]	R III/IV	ϵ_{sp} III	ϵ_{sp} III+IV	ϵ_{sp} V
Negelein	1·87	15·1	23·2	132
	1·78	—	20·6	133
Heart muscle ground with sand	1·91	18·1	27·6	171
Method A	1·97	17·3	26·1	159
($a\alpha+a\beta$)	1·94	17·0	25·8	—
	2·15	16·4	24·0	146
	1·91	16·1	24·5	149
	1·91	15·9	24·2	136
Ester	2·04	16·9	25·2	—
	1·99	15·8	23·8	138
Low R III/IV	1·60	15·5	25·2	—
fractions	1·72	13·7	21·7	—

[1] See Lemberg, Bloomfield, Caiger and Lockwood (1955).

In some of the earlier preparations, listed in Table 14, purification was carried out by repeated precipitation of crude porphyrin *a* from chloroform solution by light petroleum. This method was later abandoned when it was observed that the raspberry-red solution of porphyrin *a* in chloroform occasionally changed colour to brown or green, particularly when chloroform solutions were concentrated by evaporation at 65° C. In the experiments listed in Table 14, ϵ_{sp} was measured at 563·5 mμ in chloroform. In general, these values were lower than with the ether-acetone fractionation, the maximal value reached being 18·1.

All the differences between porphyrins *a*α and *a*β, their distribution between ether and hydrochloric acid or ether and phosphate buffer, and their adsorption to cellulose powder and to silica gel, persisted when the most highly purified preparations were compared. This excludes impurities as the cause of these differences.

After repeated attempts to crystallize various fractions from a variety of solvents, the absorption spectra of the acetone solutions were slightly altered, with R I/IV and R II/IV increased, and R III/IV slightly decreased. When the porphyrin was redissolved in secondary phosphate solution and brought back into ether by acidification, the alteration products were removed in the form of ether-insoluble flocks at the interface. Solutions of these flocks in acetone had a high R I/IV (0·47–0·49), a high R II/IV (1·33–1·54) and a low R III/IV (1·38–1·69). The porphyrin in the ether phase was pure porphyrin *a*.

A paper chromatogram according to the method of Nicholas and Rimington (1949) with 70 p.c. lutidine-30 p.c. water in an atmosphere of ammonia at 20° C. gave a spot with R_F 0·84 for "+5" porphyrin *a*β ($\epsilon_{sp}^{558} = 14·2$) as well as for "—15" ($\epsilon_{sp}^{558} = 11·0$) and for "—20" ($\epsilon_{sp}^{558} = 15·0$) porphyrin *a*$\alpha$. Since the R_F of mesoporphyrin was 0·81, that of coproporphyrin 0·62, porphyrin *a* migrates with an R_F above that found for typical dicarboxylic porphyrin, and approaches the R_F value (0·90) of a monocarboxylic porphyrin. There were also some faint spots (R_F=0·78 and 0·71 for "+5," 0·76 for "—15," and 0·71 and 0·59 for "—20" porphyrin). These experiments have been carried out by Miss J. Parker.

Porphyrin *a* methyl ester.

Porphyrin *a* is readily esterified by diazomethane in ether. The esterification is complete in a few minutes, no free porphyrin being detectable by extraction with dilute ammonia. The absorption spectrum remains unaltered. These experiments were carried out before separation of *a*α and *a*β porphyrins, but 10 p.c. HCl extracted no porphyrin ester from ether.

In contrast to the free acid porphyrin *a*, which is readily adsorbed to alumina but difficult to elute without alteration, the methyl ester—probably a dimethyl-ester, see Warburg and Gewitz (1953)—is readily fractionated on a column of

"B.D.H. aluminium oxide for chromatographic adsorption analysis." For the first studies, dry ether and ether-chloroform mixtures were used. Several fractions were obtained which differed in absorption spectrum and in their solubility in light petroleum, ether, benzene, and chloroform. However, specific extinctions were low (ϵ_{sp}^{558} = 10-12) and increases of bands I and II marked. The ester was partly soluble in light petroleum, and on evaporation the solutions in light petroleum yielded colourless crystals mixed with amorphous porphyrin *a*. These studies are therefore not reported in detail.

The results were better when the chromatography was carried out with wet ether and ether-acetone mixtures. In this way an ester of R III/IV=2·04 was obtained which had an ϵ_{sp}^{558} of 16·9, corresponding to 17·5 for the free acid. This ester was subjected by Miss Parker to the paper-chromatographic method of Chu, Green and Chu (1951). Chloroform-kerosene mixtures failed entirely to move the spot (from an ether solution); in propanol-kerosene (1:5) it moved uniformly, though with some tailing. Another preparation, of lower R III/IV (1·82) gave the same spot accompanied by one moving faster.

Dimethylacetal of ester. Esterification with methanol-hydrochloric acid transforms porphyrin *a* into a dimethyl acetal of the ester. The absorption bands are shifted towards the blue end of the spectrum and the spectrum differs from that of porphyrin *a* or its ester, in being almost an aetio-type spectrum (IV>III>II>I), with $\epsilon_{624}:\epsilon_{573}:\epsilon_{541}:\epsilon_{502}=0\cdot31:0\cdot96:1\cdot06:1$. Superficially, the reaction resembles the transformation of a phaeoporphyrin into a chloroporphyrin which occurs when the isocyclic ring of chlorophyll-porphyrins is opened by acid hydrolysis. In contrast to this reaction, however, the change of the porphyrin *a* spectrum is readily reversed. It is sufficient to allow an ether solution of the methyl acetal to stand for one hour over 5 p.c. aqueous hydrochloric acid, or to shake a chloroform solution with 20 p.c. HCl and water, to restore the typical oxorhodo-type spectrum (III>II>IV>I) and absorption bands of porphyrin *a*. This reversion can be carried out without saponification of the ester. Such alterations are due to the abolition of the formyl group —C=O double bond by its transformation into a dimethyl acetal (cf. Fischer and Wecker, 1941).

Oxime of porphyrin *a*.

Porphyrin *a* (R III/IV=1·96) was converted into its oxime by heating of its pyridine solution for a few minutes with a mixture of equivalent amounts of hydroxylamine hydrochloride and sodium carbonate, or of hydroxylamine hydrochloride and sodium acetate. The oxorhodo-type spectrum of porphyrin *a* changed into a spectrum with absorption bands at 644, 577, 553 and 512 mμ, and an additional weak band at 664 mμ. The spectrum in ether was: $\epsilon_{664}:\epsilon_{643}:\epsilon_{578.5}:\epsilon_{554.5}:\epsilon_{513.5}=0\cdot23:0\cdot26:1\cdot0:1\cdot36:1\cdot0$. After removal of the pyridine with 1 p.c. HCl, 4 p.c. HCl extracted the pure oxime which no longer

had a band at 664 mμ and had a typical rhodo-type spectrum ($\epsilon_{642}:\epsilon_{587}:\epsilon_{548.5}:\epsilon_{508}$ =0·20:0·67:1·34:1, III>IV>II>I). Stronger HCl extracted more oxime, but also the compound with the absorption band at 664 mμ. Our oxime resembles that obtained by Rawlinson and Hale (1949) except for its somewhat higher R III/IV (1·34 instead of about 1·23). It differs from the oxime obtained by Dannenberg and Kiese (1952) from a porphyrin *a* of R III/IV of 1·6 by having a rhodo-type spectrum in ether, whereas the oxime of these authors had an aetio-type spectrum in ether, though a rhodo-type spectrum in chloroform; our oxime resembles the oxime later obtained by Kiese (1952) from a porphyrin *a* of R III/IV 1·75 in its band positions in pyridine.

Haemin *a*.

Preparation by re-introduction of iron into porphyrin a. Iron is readily re-introduced into porphyrin *a* by heating the porphyrin solution in glacial acetic acid at 85° C. with ferrous acetate and dilute hydrochloric acid. The solution turns red-brown and an absorption band at about 660 mμ appears. The greatest increase of absorption was found at 665 mμ, the greatest decrease at 558 mμ, and a ratio of $\epsilon_{665}/\epsilon_{558}$ was therefore used for measuring the completeness of the reaction. This ratio is 0·025 for porphyrin *a* and rises to a limiting value of 0·38 for haemin *a*.

For the preparation of the purest haemin *a* an ether solution of porphyrin *aa* (R III/IV=1·98) was used which had been purified by chromatography on cellulose powder and on silica gel, but which had not been subjected to solvent fractionation of the solid porphyrin, and therefore still contained some lipoid. Ether solutions containing about 1 mg. of porphyrin *a* were evaporated in vacuo, washed with light petroleum and dissolved in 10 ml. glacial acetic acid to which 0·1 ml. saturated aqueous sodium acetate solution was added. The solution was rapidly heated to 85° C. by immersion in a hot water bath, and after the addition of 0·2 ml. of a solution containing 5 gm. $FeSO_4 \cdot 7H_2O$ in 100 ml. water, and of 0·3 ml. 1 p.c. HCl, heating was continued for 5 minutes. The solution was then rapidly cooled and $\epsilon_{665}/\epsilon_{558}$ measured. This had risen to 0·37. The same result was obtained when the amount of iron was doubled. The molar ratios porphyrin:Fe:sodium acetate:HCl were 1:3·2:200:9. The absorption spectrum of the acetic acid solution showed a plateau at 650–660 mμ, and two maxima at 554 and 510 mμ. The solution was diluted with ether and the major part of the acetic acid removed by washing with distilled water. The spectrum of acid haematin in this ether-acetic acid solution was now:

λ in mμ:	655 (max.)	635 (min.)	552·5 (max.)	528 (min.)	510 (max.)	475 (min.)
ε (relative)	0·415	0·39	1·0	0·79	0·85	0·72

The ether solution was now shaken with M/5 phosphate buffer of pH 6·5 to extract the remainder of the acetic acid. The colour changed from red-brown

to green and flocks of the neutral haematin were precipitated at the interface; at this stage the ether was in equilibrium with a buffer of pH 5·2. Precipitation was never complete. The haematin precipitate was collected on a small filter, washed with distilled water, dissolved in dilute ammonia and brought into fresh ether as haemin by acidification with hydrochloric acid. This haemin *a* preparation (A) was amorphous, insoluble in light petroleum, but readily soluble in ether or acetone containing traces of hydrochloric acid. The ether solution remaining on washing with phosphate buffer gave a less pure preparation of haemin *a*; on evaporation of the haemin solution the solid contained some colourless material which was not quite as readily soluble in acetone as the haemin, and probably contained some phospholipid.

Other preparations were obtained in a similar way from porphyrin *a* of ϵ_{sp}^{558} of 15–16 without precipitation from ether by neutralization. They gave pyridine haemochromes of lower specific extinction than preparation A.

Absorption spectra. The absorption spectrum of haemin *a* in ether-HCl is the same as that described above for the ether-acetic acid solution.

The ether solution of the green alkaline haematin *a*, probably the [FeOH] compound, has a strong absorption band at 625 mμ and a weaker band at 570 mμ with absorption minima at 605 and 530 mμ. It is readily converted into the chlorohaemin by shaking the green ether solution with sodium chloride solution, when it turns red-brown and displays the haemin spectrum. The hydroxyl group is so readily dissociated that great dilution of the green alkaline haematin solution with ether dissociates it almost entirely. This gives a change in colour to red-brown, and a change in spectrum to that of acid haematin, identical with that of the haemin.

In 0·01–0·1 N NaOH the alkaline haematin has a distinct single absorption band at 639 mμ, with a minimum of absorption at about 570 mμ, and gradual rise towards the ultraviolet. In contrast to protohaematin in alkaline solution, haematin *a* does not react with cyanide; the spectrum remains unchanged.

Haem, Haem-CN, Haem-CO. Dithionite reduces the alkaline haematin to haem *a* which has a single absorption band at 606 mμ in 0·01 N NaOH with a minimum of absorption at 515 mμ. In contrast to the haematin, the haem reacts with cyanide. The ferrous cyanide compound has a strong absorption maximum at 598 mμ, a weaker one at 533 mμ and a Soret band at 448 mμ, which is only 4·36 times as strong as the band at 598 mμ. Carbon monoxide gives a similar absorption spectrum with bands at 602, (535) and 423 mμ, with a ratio of $\epsilon_{432}/\epsilon_{602}$ of 4·7.

Pyridine haemochrome. The pyridine haemochrome has a weak absorption band at 587 mμ which is greatly increased by reduction with sodium dithionite to the haemochrome. The haemochrome has a strong absorption band at 587 mμ and a Soret band at 430 mμ which is about 4·6 times as strong as the band at 587 mμ. The details of its spectrum, however, depend on the conditions to a

far greater extent than the absorption spectrum of protohaemochrome, and this has led to some confusion. Thus Warburg and Gewitz (1951) reported that a band resembling in position that of protohaemochrome is formed on reduction with dithionite, while no such band was observed by Rawlinson and Hale (1949), Lemberg (1935) or Kiese and Kurz (1954); again Kiese and Kurz observed a weak band at 534 mμ, not found by the other authors. The experiments described below show that the occurrence of these bands depends on the nature of the solvent and other conditions.

A solution of preparation A of haemin *a* in 50 (v/v.) aqueous pyridine was reduced with a small amount of solid sodium dithionite, and the absorption at the maximum (587 mμ) measured at 1 minute intervals. The absorption rose rapidly to a maximum in about 3 minutes ($\epsilon_{sp}^{587} = 32{\cdot}0$), and then decreased very slowly by about 0·3 p.c. per minute, the absorption at 558 mμ decreasing in the same proportion as that at 587 mμ. A weak maximum at 535 mμ and a minimum at 545–550 mμ were observed, but no band at 558 mμ. The ratio $\epsilon_{587}:\epsilon_{558}:\epsilon_{535}$ was 1:0·39:0·40, similar to that found by Kiese and Kurz. A different spectrum was, however, obtained when the solution contained 0·1 p.c. NaOH in addition to 5 p.c. pyridine. The specific extinction at 587 mμ was somewhat higher (33·5) and decreased as slowly as in the neutral solution. The band at 587 mμ was the only one observable in the visible part of the spectrum. Instead of the 535 mμ band in neutral 50 p.c. pyridine, the absorption rose quite steadily from the minimum at 545 mμ towards the Soret band. The ratios were $\epsilon_{587}:\epsilon_{558}:\epsilon_{535}=1:0{\cdot}44:0{\cdot}39$. Even ammonia or phosphate buffer added to the 50 p.c. pyridine solution abolished the weak 535 mμ band. 20 p.c. pyridine containing 1·0 p.c. or 0·1 p.c. NaOH gave the same results. Under all these conditions $\epsilon_{558}/\epsilon_{587}$ was low (0·37–0·45). Under other conditions, however, particularly in a weak pyridine solution, e.g., in 3 p.c. pyridine which Warburg apparently used (see Warburg, 1954), the protohaemochrome-like band was formed with varying rapidity; this was also found when pyridine was replaced by 20 p.c. ammonia or dithionite by ascorbic acid. The ratio $\epsilon_{558}/\epsilon_{587}$ was then noticeably higher (0·5–0·6), and the extinction at 587 mμ depressed by 10–30 p.c. On long standing of the reduced solutions in air, an absorption band at 560 mμ appeared slowly even in stronger pyridine.

The above high values for ϵ_{sp}^{587} were obtained only from preparation A of the haemin. Other preparations gave lower values, which under the optimal conditions described above varied from 21·0 to 24·5.

Reconversion of haemin a *into porphyrin* a. The reconversion of haemin *a* into a porphyrin *a* with the same high R III/IV as that from which the haemin had been formed, has already been discussed by Lemberg, Bloomfield, Caiger and Lockwood (1955). An unusual splitting of iron from the haemin was observed. In an attempt to crystallize haemin *a* from its solution in acetone containing traces of hydrochloric acid by the addition of light petroleum, an amorphous

precipitate of haemin *a* was obtained. The solution was filtered and allowed to stand for several days at room temperature. Its colour had then become raspberry-red and the spectrum was that of porphyrin *a*. Short straight needles had crystallized on the vessel walls. These were dissolved in secondary phosphate and driven into ether after acidification with dilute phosphoric acid. The spectrum was a typical porphyrin *a* spectrum with R III/IV=2·05, and $\epsilon_{665}/\epsilon_{558}=0{\cdot}04$.

Comparison of haemin a *reconstituted from porphyrin* a *with haemin* a *isolated by the method of Kiese and Kurz (1954).* It was of interest to compare our haemin *a* reconstituted from porphyrin *a* with haemin *a* isolated directly. No details have yet been published on the preparation of crystalline haemin *a* by Warburg and Gewitz (1951), nor have they published spectroscopic data. We have, however, repeated the method of Kiese and Kurz (1954) by which haemin *a* is separated from protohaemin by alumina chromatography and particularly by countercurrent distribution between a light petroleum-acetone and an aqueous acetone-HCl phase. We have been unable to obtain anything approaching the 70–80 p.c. yield claimed by the authors and the haemin *a* still contained much colourless lipoid.

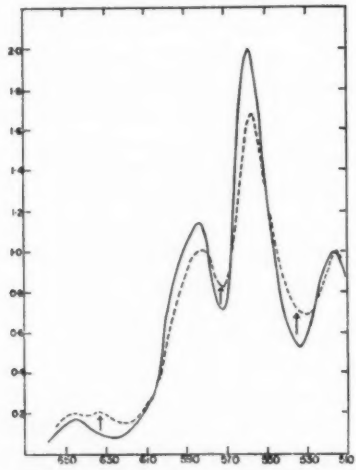

Fig. 5. Absorption spectrum of porphyrin *a* from haemin prepared according to Kiese and Kurz (1954) - - - - -.

Compared with pure porphyrin *a* ———.

Arrows refer to maxima of difference of absorption corresponding to the absorption maxima of protoporphyrin.

The ϵ_{sp}^{587} of the pyridine haemochrome, which was readily soluble in light petroleum, was only 4·2 in neutral 50 p.c. pyridine ($\epsilon_{587}:\epsilon_{558}:\epsilon_{535}=1:0{\cdot}44:0{\cdot}49$) with a weak band at 535 mμ. It was 5·0 in 50 p.c. pyridine containing 0·01 p.c. NaOH, when the 535 mμ band was absent as in our preparation.

After removal of iron by the ferrous sulphate-HCl method of Lemberg, Bloomfield, Caiger and Lockwood (1955), p. 444, the presence of protoporphyrin could readily be demonstrated (see Fig. 5).

The regions marked by an arrow in which the absorption was higher than that of pure porphyrin *a* correspond exactly to the maxima of absorption of protoporphyrin. The ratio R III/IV was only 1·69 and could be raised to 1·87 by extraction of protoporphyrin with 8 p.c. HCl, while the spectrum in ether of the porphyrin recovered from the 8 p.c. HCl extract was predominantly that of protoporphyrin with a little cryptoporphyrin. According to the spectrophotometric analysis by Lemberg and Parker (1955), this fraction contained 61 p.c. protoporphyrin, 21 p.c. of cryptoporphyrin *a* and only 18 p.c. of porphyrin *a*. Thus, the haemin *a* obtained by us with the method of Kiese and Kurz was not quite free from protohaemin.

Copper and Zinc Complexes of Porphyrin *a*.

The copper complex of porphyrin *a* in ether-acetic acid has the spectrum described by Rawlinson and Hale (1949), with absorption bands at 593·5, 548 and 525 mμ (I>II>III). The spectrum of the zinc complex in the same solvent showed a strong band at 605·8 mμ and a weak band at 564·1 mμ. The chlorin, which had been obtained as a product of alteration of porphyrin *a* $\alpha\beta$ gave a copper complex with a strong band at 646 mμ, and weaker ones at 586, 562 and 526 mμ.

DISCUSSION.

The differences previously observed between our haemin *a* preparations and those by Warburg and Gewitz, and by Kiese, have been shown above to be due to differences in experimental conditions, and do not exclude identity. The only difference which remains so far unexplained is the great solubility of even our purest haemin *a* in acetone containing hydrochloric acid, while Warburg and Gewitz (1951) reported, without giving details, that their haemin *a* crystals had been obtained from this medium. The specific extinction coefficient at 587 mμ of our purest pyridine haemochrome *a* (32·0) reconstituted from porphyrin *a*, in conjunction with the millimolar extinction coefficient (28·2), calculated from its iron content (Dannenberg and Kiese, 1952), gives a molecular weight of 880 for our haemin. This is in good agreement with the figure of 870 calculated from the iron content of the crystals prepared by Warburg and Gewitz. This preparation of ours was thus at least 99 p.c. pure, but other preparations of the haemin reached only a purity of 65–75 p.c.

Although only in one instance good crystals of porphyrin *a* have been obtained, namely from the above mentioned haemin *a* preparation, the spectrophotometric data show that our purest amorphous preparations were of almost the same degree of purity. Assuming the molecular weight of porphyrin *a* to be 780, and the sum of the millimolar extinction coefficients at the maxima of bands III and IV in organic solvents to be 25·4, as are those of the related chlorocruoroporphyrin as well as the average values of numbers of related porphyrins (Lemberg, 1953), our purest porphyrin would be 93 p.c. pure. The same value is reached by using the figure 16·9 for ϵ_{mM} of the maximum of band III (Oliver and Rawlinson, 1951). Purities of about 90 p.c. have regularly been obtained in our preparations. While a purity of 70–75 p.c. is easily achieved with excellent yield, the last trace of lipoids is tenaciously held. The long alkyl side chain of the molecule (Warburg and Gewitz, 1951; Lemberg, 1953) explains this by the strong van der Waals forces between this group and similar groups in lipoids, as well as the fact that porphyrin *a* and haemin *a* are more lipophilic than other porphyrins and haemins. This is also probably of fundamental importance for the structure of mitochondria, in which the haem *a* proteins are closely linked with phospholipids.

The chemical structure of porphyrin *a* is still not fully explored, and the observations described in this paper, particularly the existence of two spectroscopically identical and interconvertible porphyrins a_α and $a\beta$ cannot readily be explained on the basis of the formula suggested so far, e.g., on that by Warburg and Gewitz (1953) and Warburg (1954).

There is good evidence for the presence of a formyl side chain (Lemberg and Falk, 1951; Lemberg, 1953). The dimethylacetal formation described in this paper further supports this. It disproves the assumption of an isocyclic ring, like that found in chlorophyll. Infra-red spectra of porphyrin *a* (Willis and Lemberg, unpublished) also show the carbonyl group to be formyl (or acetyl), but not a ring keto group.

Porphyrin *a* can scarcely be a phytol ester since porphyrin *a* not exposed to strong hydrochloric acid is insoluble in light petroleum, and can be purified chromatographically and by solvent fractionation to the same degree of high extinction as porphyrin *a* treated with hydrochloric acid under conditions under which a phytol ester would be hydrolysed. Porphyrin *a* is also soluble in alkaline phosphate, though this would not exclude a monophytol ester of a dicarboxylic acid.

The second group whose presence transforms the normal rhodo-type spectrum of formylporphyrins into an oxorhodo-type spectrum (Lemberg, 1953) very likely contains a double bond conjugated with the porphyrin ring, in such a position that it increases the rhodofying effect of the formyl group. The group still exerts a distinct rhodofying effect when the formyl group is transformed into the —CH=NOH group (oxime), or into the dimethyl acetal. Under other

conditions, however, it appears to be more labile than would be expected for a vinyl group, and the decrease of R III/IV, frequently mentioned above, is due to alteration of this group with abolition of its rhodofying effect. Lemberg and Falk (1951) assumed this group to be an acrylic acid side chain, but recent measurements of the infra-red absorption of porphyrin *a* methyl ester (Willis and Lemberg, unpublished) make this assumption unlikely. According to Warburg and Gewitz (1953) it is a double bond connecting the long alkyl group to the porphyrin ring, and is removed with the alkyl group in the resorcinol melt of the haemin. These authors assume that the CHO and the —CH=CHR groups are present in positions 2 and 3 of the porphyrin ring, i.e., on proximal pyrrole rings. In porphyrins with two rhodofying groups in position 2–4 (or 1–3), e.g., diformyldeuteroporphyrin, as well as in porphyrins with such groups in positions 1 and 4 (porphyrins derived from chlorophyll b), the two groups counteract each other so that the spectrum is of aetio-type. It seems therefore improbable that the two groups in the 2 and 3 positions should reinforce their effect. It appears more likely that the two groups are on opposite pyrrole rings, e.g., I and III, and the only oxorhodo-type porphyrins so far known are of this type (Lemberg, 1953); or that the two groups are on one and the same pyrrole, but such compounds are not yet known.

While Falk and Rimington (1952) believed they had evidence that the removal of the iron causes an irreversible alteration of porphyrin *a*, we have shown that unaltered porphyrin *a* can be obtained from haemin *a* and can be reconverted into intact haemin *a*.

Porphyrin *a* has been obtained in two interconvertible forms, porphyrin *aα* and *aβ*, which differ in several of their properties, but have the same absorption spectra. The fact that the absorption spectra do not differ means that the difference between the two porphyrins, and the reactions transforming one into the other must involve groups which do not belong to the resonating system, or if they involve such groups must be of a nature which does not greatly affect the resonance system. The only way in which such an alteration may be explained on the basis of the formula of Warburg and Gewitz, would be *cis-trans* isomerism on the double bond connecting the long alkyl group with the porphyrin ring; such an explanation does not appear likely in view of the nature of the differences between porphyrin *aα* and *aβ*.

A hydroxyl group may be present on the long alkyl group and this is supported by the solubility of porphyrin *a* in alcohols. If this is so, lactone ring formation between this group and one of the carboxyl groups may be involved, but the stability of both forms towards alkali does not speak in favour of such an assumption.

There are two other aspects in which these findings are of interest. The possibility of obtaining porphyrin *a* in several fractions may have indicated that porphyrin *a* is in fact composed of a variety of similar porphyrins differing

in groups which would not influence the absorption spectrum, for example, the long alkyl side chains. Our observations while not rigidly excluding the possibility that several very closely related porphyrins *a* exist, do not lend support to this. They indicate that only two forms, porphyrins a_α and $a\beta$, are obtained and that these are interconvertible.

The second point of interest is the possible relationship between these two porphyrins and the two cytochromes *a* and a_3. Our findings leave this possibility open, since in whatever form, a_α, $a\beta$ or $a_\alpha+a\beta$, the porphyrin may be in the cytochromes, it would be changed into a_α during the process of removal of the iron, the form which alone is found when further alterations by aqueous hydrochloric acid at room temperature are avoided.

The observations on the absorption spectra of the haem *a* compounds are of some interest with regard to the spectroscopic properties of the cytochromes *a*, a_3 and a_1. The position of the absorption maximum of haem *a* at 606 mμ resembles that of cytochrome *a*. Another similarity is the relatively low height of the Soret band, which is only 4·7 times as strong as the band in the yellow for the CO-compound, and 4·6 for the pyridine haemochrome. However, haem *a* forms a CO compound—cytochrome *a* does not. Another point of interest is that haematin *a* in alkaline solution differs from protohaematin *a* in that it does not react with cyanide. The alkaline haematin band at 639 mμ is not shifted by cyanide. Warburg and Negelein (1933) had observed a band at 639 mμ in acetic acid bacteria in the presence of cyanide, which has been repeatedly discussed (cf. Lemberg and Legge, 1949). It now appears likely that this band was simply alkaline haematin *a* produced by the alkalinity of the cyanide. The fact that no reaction between alkaline haematin *a* and cyanide takes place may be cited in connection with the fact that it is HCN, not the cyanide ion, which inhibits cytochrome oxidase (Horecker and Stannard, 1948). In contrast to haematin *a*, haem *a* reacted with cyanide. Further investigations on synthetic haem *a* proteins are being carried out which promise to throw more light on these problems.

SUMMARY.

Methods are described for the purification of porphyrin *a* from heart muscle. After removal of protoporphyrin and a little cryptoporphyrin *a* by extraction from ether with 8 p.c. HCl, porphyrin *a* is further purified from tenaciously held lipoids by chromatography on cellulose powder and on silica gel columns, and by fractional extraction with solvents. This gives porphyrin a_α. By treatment with aqueous hydrochloric acid, porphyrin a_α is partly converted into porphyrin $a\beta$ which has the same absorption spectrum, but differs from porphyrin a_α in its distribution between ether and hydrochloric acid, ether and phosphate buffer, and in its chromatographic behaviour. Porphyrins a_α and $a\beta$ are separated by

these methods, and both are obtained in the same high degree of purity. Both are stable to alkali.

Haem *a* is reconstituted from porphyrin *a* by introduction of iron, and the iron removed again without alteration of the spectroscopic properties of porphyrin *a*. Porphyrin *aβ* is, however, converted to porphyrin *aa* during the removal of iron. The problem of whether the existence of the two interconvertible forms is related to the existence of the two cytochromes, *a* and a_3, in heart muscle, must therefore remain open.

Crystalline porphyrin *a* was obtained from reconstituted haemin. The properties of porphyrin *a* and several of its compounds, including some haematin *a* compounds, are described.

The relevance of these observations to the problem of the structure of porphyrin *a*, to the properties of cytochrome *a* and cytochrome oxidase, and to the incorporation of the latter in mitochondria are discussed.

REFERENCES.

Bush, M. T. and Densen, P. M. (1948): Anal. Chem., 20, p. 21.
Chu, T. C., Green, A. A. and Chu, E. J.-H. (1951): J. biol. Chem., 190, p. 643.
Dannenberg, H. and Kiese, M. (1952): Biochem. Z., 322, p. 395.
Falk, J. E. and Rimington, C. (1952): Biochem. J., 51, p. 36.
Fischer, H. and Wecker, G. (1942): Z. physiol. Chem., 272, p. 1.
Granick, S. and Bogorad, L. (1953): J. biol. Chem., 202, p. 781.
Horecker, B. L. and Stannard, J. N. (1948): *Ibid.*, 172, p. 599.
Kiese, M. (1952): Naturwiss., 39, p. 403.
Kiese, M. and Kurz, H. (1954): Biochem. Z., 325, p. 299.
Lemberg, R. (1953): Nature, 172, p. 619.
Lemberg, R. (1955): Ann. Acad. Sci. Fenn., Ser. A II, Chemica, N: 60.
Lemberg, R., Bloomfield, B., Caiger, P. and Lockwood, W. H. (1955): Austral. J. exp. Biol., 33, p. 435.
Lemberg, R. and Falk, J. E. (1951): Biochem. J., 49, p. 674.
Lemberg, R. and Legge, J. W. (1949): Hematin Compounds and Bile Pigments, Interscience Publishers, New York, p. 365.
Lemberg, R. and Parker, J. (1952): Austral. J. exp. Biol., 30, p. 163.
Lemberg, R. and Parker, J. (1955): *Ibid.*, 33, p. 483.
Lucas, J. and Orten, J. M. (1951): J. biol. Chem., 191, p. 287.
Negelein, E. (1933): Biochem. Z., 266, p. 412.
Nicholas, R. E. H. and Rimington, C. (1949): Scand. J. clin. Lab. Invest., 1, p. 12.
Oliver, I. T. and Rawlinson, W. A. (1951): Biochem. J., 49, p. 157.
Rawlinson, W. A. and Hale, J. W. (1949): *Ibid.*, 45, p. 247.
Stern, A. and Molvig, H. (1936): Z. physikal Chem., A 177, p. 365.
Stern, A. and Wenderlein, H. (1934): *Ibid.*, A 170, p. 345.
Warburg, O. (1954): Naturwiss, 40, p. 493.
Warburg, O. and Gewitz, H. S. (1951): Z. physiol. Chem., 288, p. 1.
Warburg, O. and Gewitz, H. S. (1953): *Ibid.*, 292, p. 174.
Warburg, O. and Negelein, E. (1933): Biochem. Z., 262, p. 237.

PORPHYRINS WITH FORMYL GROUPS VII

SPECTROPHOTOMETRIC ANALYSES OF MIXTURES OF PROTOPORPHYRIN, PORPHYRIN *a* AND CRYPTOPORPHYRIN *a*

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The porphyrins which are obtained from the haematin compounds of heart muscle consist of two major components, protoporphyrin and porphyrin *a*, and a third present in varying amounts, cryptoporphyrin *a* (Lemberg, Bloomfield, Caiger and Lockwood, 1955). Possibly other porphyrins, such as haematoporphyrin and related porphyrins (Granick, 1952), and cryptoporphyrin *p* (Lemberg, 1953), are also present; these as well as cryptoporphyrin *a* may be artifacts.

An attempt to devise a method for spectrophotometric estimation of the porphyrins in mixtures appeared to be worthwhile for several reasons. While the complete separation of porphyrin *a* from protoporphyrin derived from heart muscle has been achieved without substantial loss (Lemberg, Bloomfield, Caiger and Lockwood, 1955), the separation has been less successful with extracts from other organs and micro-organisms (Lemberg, Stewart and Bloomfield, 1955). Secondly, cryptoporphyrin *a* is difficult to separate in quantitative yield from protoporphyrin; protoporphyrin is present in far higher concentration than cryptoporphyrin so that a substantial part of cryptoporphyrin may escape discovery in the protoporphyrin fraction. In fact, the presence of cryptoporphyrin in heart muscle extracts was discovered by a spectrophotometric investigation (Lemberg, 1953). A spectrophotometric analysis for mixtures of all three porphyrins would also be useful for checking the efficiency of separation procedures.

For such an analysis the absorption spectra and extinctions of the porphyrins must be known. We have so far studied only protoporphyrin, porphyrin *a* and cryptoporphyrin *a*, and have neglected other porphyrins possibly present in small amounts. The absorption spectrum of protoporphyrin is well known; that of porphyrin *a* is described in a preceding paper of this series (Lemberg and Stewart, 1955). Isolation and properties of cryptoporphyrin *a*, briefly described

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by Lemberg (1953), will be published in detail by one of us (J.P.) elsewhere. We make use here of the knowledge of its absorption spectrum and the extinctions. Some revision may be necessary for the values of the specific extinction coefficients of cryptoporphyrin *a* which were derived from one preparation of crystalline dimethyl ester of m.p. 259–60° C. Since this was spectroscopically pure, the revision would only affect the amounts of cryptoporphyrin *a*. These data allow us to choose suitable wavelengths and to develop the formulae for the spectrophotometric analysis.

On the whole, this analysis works satisfactorily; contradictory results obtained in some experiments are an indication of the presence of minor amounts of other porphyrins. Later it will be attempted to establish a similar method for the haemins in order to detect alterations during the removal of iron. Our experience has convinced us, however, that the separation of the haemins themselves is difficult to achieve, and that the haemins must be reconstituted from the pure porphyrins.

EXPERIMENTAL.

Four wavelengths (580, 555, 538, 513 mμ) have been chosen for the analysis. At 538 mμ, protoporphyrin has its absorption maximum III, whereas the two formylporphyrins, porphyrin *a* and cryptoporphyrin *a*, have a low absorption in this region. The wavelength 555 mμ is close to the maxima of absorption of the two formylporphyrins (558 mμ and 554–5 mμ respectively), and their specific extinctions at 555 mμ (see Table 1) are almost equal. The ratio $\epsilon_{555}/\epsilon_{538}$ is 0·18 for protoporphyrin, 1·96 for cryptoporphyrin *a* and 3·18 for porphyrin *a*. The wavelength 513 mμ is close to the maxima IV of the three porphyrins (porphyrin *a* 516 mμ, cryptoporphyrin 513 mμ, and protoporphyrin 503 mμ), and there is a large difference here in absorption between porphyrin *a* and cryptoporphyrin *a*. Finally, at 580 mμ the absolute values of the extinctions of the three porphyrins are similar, but while the ratio $\epsilon_{580}/\epsilon_{513}$ for protoporphyrin and cryptoporphyrin *a* is almost equal (0·66 and 0·64), that for porphyrin *a* is much higher. It was this observation that showed that the 8 p.c. hydrochloric acid extracts of the heart muscle porphyrins did not contain a mixture of protoporphyrin and porphyrin *a*, for while the ratio $\epsilon_{555}/\epsilon_{538}$ would have supported such an assumption, the ratio $\epsilon_{580}/\epsilon_{513}$ was that of pure protoporphyrin. The use of four parameters for the estimation of three unknowns gives an internal check to the method.

The specific extinction coefficients of the three porphyrins at the four wavelengths are given in Table 1.

Three specimens of protoporphyrin all agreed closely with each other at these wavelengths, and agreed in their extinction ratios at the maxima with those given by Stern and Wenderlein (1954).

TABLE 1.

Specific extinction coefficients of protoporphyrin, porphyrin *a*, and cryptoporphyrin *a*. Solvent: ether—acetic acid.

λ	Protoporphyrin	Porphyrin *a*	Cryptoporphyrin *a*
580	9·9	11·5	11·1
555	3·44	19·5	19·4
538	19·1	6·14	9·9
513	15·05	9·7	17·4

If ϵ_λ means extinction (D/1, 1 in cm.) at wavelength and $E=\epsilon_\lambda v$ (v=volume in ml.), the following equations are derived:

(a) for mixtures of protoporphyrin and cryptoporphyrin containing no porphyrin *a*:
mg. cryptoporphyrin=$0{\cdot}0567\ E_{555}-0{\cdot}0102\ E_{538}$
mg. protoporphyrin=$0{\cdot}0577\ E_{538}-0{\cdot}0295\ E_{555}$

(b) for mixtures of protoporphyrin and porphyrin *a* containing no cryptoporphyrin:
mg. porphyrin *a*=$0{\cdot}0543\ E_{555}-0{\cdot}0098\ E_{538}$
mg. protoporphyrin=$0{\cdot}0555\ E_{538}-0{\cdot}0175\ E_{555}$

(c) for mixtures of porphyrin *a* and cryptoporphyrin containing no protoporphyrin:
mg. porphyrin *a*=$0{\cdot}115\ E_{555}-0{\cdot}128\ E_{513}$
mg. cryptoporphyrin=$0{\cdot}129\ E_{513}-0{\cdot}064\ E_{555}$

(d) for mixtures containing all three porphyrins, using ϵ_{555}, ϵ_{538} and ϵ_{513}:
mg. porphyrin *a*=$0{\cdot}106\ E_{555}+0{\cdot}133\ E_{538}-0{\cdot}193\ E_{513}$
mg. cryptoporphyrin=$0{\cdot}202\ E_{513}-0{\cdot}149\ E_{538}-0{\cdot}053\ E_{555}$
mg. protoporphyrin=$0{\cdot}0871\ E_{538}-0{\cdot}0426\ E_{513}-0{\cdot}0062\ E_{555}$

(e) for mixtures containing all three porphyrins, using ϵ_{580}, ϵ_{555} and ϵ_{513}:
The formula can be somewhat simplified, since ϵ_{sp} of porphyrin *a* and cryptoporphyrin at 580 and 555 mμ differ by little more than the experimental error.
mg. (porphyrin *a*+cryptoporphyrin)=$0{\cdot}0645\ E_{555}-0{\cdot}0225\ E_{580}$
mg. porphyrin *a*=$0{\cdot}186\ E_{580}+0{\cdot}003\ E_{555}-0{\cdot}123\ E_{513}$
mg. protoporphyrin=$0{\cdot}126\ E_{580}-0{\cdot}073\ E_{555}$

RESULTS.

In this section a few applications of the methods are given, sufficient to show their usefulness, and at the same time to reveal their limitations.

TABLE 2.

Ether solutions of mixtures of the porphyrins analysed by method (e) at 580, 555 and 513 mμ.

		Porphyrin *a* mg.	Protoporphyrin mg.	Cryptoporphyrin *a* mg.
Crude ether extract of heart porphyrins	a[1]	0·65	0·81	−0·081
	b	0·60	0·78	−0·034
	c	0·62	0·77	−0·016
After 3 p.c. HCl	a	0·665	0·07	−0·005
	b	0·58	0·11	−0·003
After 8 p.c. HCl	a	0·56	0·04	+0·024
	b	0·555	0·00	+0·031

[1] a, b and c are replicates of porphyrin solutions from the same preparation of crude haemin.

In the first experiments (Table 2), three samples of the crude ether extract of the heart muscle porphyrins obtained from the same amount of crude haemin are analysed before fractionation, after extraction with 3 p.c. HCl, and again after extraction with 8 p.c. HCl. The method gives reasonable results for porphyrin *a* and for protoporphyrin. Little porphyrin *a* is extracted by either 3 p.c. or 8 p.c. HCl, while the main part of protoporphyrin is extracted by 3 p.c. HCl and the remainder by 8 p.c. HCl. The results for the small amounts of cryptoporphyrin are less reliable. Small negative values for cryptoporphyrin are found in the crude extract, but small positive values after extraction with 8 p.c. HCl. This gives the impression, not supported by other evidence, that some cryptoporphyrin is formed during the extraction from ether with dilute hydrochloric acid. The negative values for cryptoporphyrin in the crude extract make it appear more likely that the change, if in fact it be outside the experimental errors of the method, has to be explained by the extraction by hydrochloric acid of a porphyrin other than those accounted for in the method, rather than by the formation of cryptoporphyrin.

For the purpose of estimation of cryptoporphyrin it is thus necessary to investigate the ether solutions recovered from the 3 p.c. HCl extract (protoporphyrin fraction) and particularly the 8 p.c. HCl extract (cryptoporphyrin fraction). Table 3 gives a few typical experiments representative of many similar ones which have been carried out. The cryptoporphyrin fraction (lines 4 and 5) still contains some protoporphyrin besides cryptoporphyrin which forms 60 p.c. (in other experiments up to 90 p.c.) of the porphyrin. Repeated extractions with 4 p.c. HCl remove the protoporphyrin entirely (line 6), leaving prac-

TABLE 3.

Ether solutions of mixtures of protoporphyrin (Pr), porphyrin *a* (P*a*), and cryptoporphyrin *a* (Cr) analysed by different methods.

Percent of total porphyrin.

	Method (a)		Method (d)			Method (e)		
	Pr	Cr	Pr	Cr	P*a*	Pr	Cr	P*a*
Collected proto fractions of heart	90·5	9·5	89	25	−14	91	13	−4
Proto fraction A	98·2	1·8	94	18·5	−12·5	100	−6	6
Proto fraction B	97·3	2·7	97·5	19·5	−17	100·8	1·1	−1·9
Crypto fraction A	40	60	39	64	−3	45	46	9
Crypto fraction B	41	59	45	56·5	−1·5	50	42	8
Crypto refractionated	1	99*	0	105·5	−5·5	3	106·5	−9
8 p.c. HCl extract	90	10	89	19	−8	89·5	16·5	−6
(proto + crypto)	78	22	79	20·5	0·5	79	13·5	7·5

* By method (c): 94 p.c. Cr, 6 p.c. P*a*.

tically pure cryptoporphyrin. The values obtained by the three methods (a), (d) and (e) for cryptoporphyrin and protoporphyrin agree moderately well, except that the cryptoporphyrin values by method (e) are somewhat smaller and the protoporphyrin values somewhat larger than by the two other methods; and that method (e), but not method (d), indicates the presence of some porphyrin *a*. Porphyrin *a*, if present at all in the cryptoporphyrin fraction, forms only a very small part. The differences between the methods (d) and (e) may be due to the presence of some cryptoporphyrin *p* whose spectrum would qualitatively fit the observed differences. The analysis of the ether solution of the cryptoporphyrin *a* (line 6) indicates that the impurity is removed with the protoporphyrin. The findings in the protoporphyrin fraction (lines 1–3, Table 3) indeed show that the impurity is also present in it. Again, cryptoporphyrin values by method (d) are higher than by method (e) and here even higher than by method (a), and negative values for porphyrin *a* are obtained by method (e).

All three methods give similar high values for the protoporphyrin content (90–100 p.c. of the fraction). Since porphyrin *a* is absent, method (a) analyzing only for mixtures of proto- and cryptoporphyrin is preferable. Although the amount of cryptoporphyrin in the protoporphyrin fractions is very small compared with protoporphyrin, it is not negligible in comparison with the total cryptoporphyrin in the sum of the two fractions. In experiment A (lines 2 and 4) 24 p.c., in experiment B (lines 3 and 6) 15 p.c., and in a third experiment 45 p.c. of the total cryptoporphyrin was found in the protoporphyrin fraction.

In the experiments of lines 7 and 8 of Table 3 protoporphyrin and cryptoporphyrin were extracted together by 8 p.c. HCl. As expected, the results are between those in which protoporphyrin and cryptoporphyrin were separated. If only an estimation of cryptoporphyrin, not its isolation, is required this is the simplest procedure, and most of the calculations of yield of cryptoporphyrin in the following section have been obtained by it, using method (a) based on measurements of extinction at 555 and 538 mμ.

TABLE 4.

Porphyrin *a* of low R III/IV analysed by methods (c) and (e).
Pa = porphyrin *a*, Pr = protoporphyrin, Cr = cryptoporphyrin *a* in mg.

Porphyrin	Method (c)	Method (e)		
	Cr	Pa	Pr	Cr
"−10", R III/IV = 1·77	0·52	2·36	0·21	0·23
"−20", R III/IV = 1·93	0·18	3·12	0·13	0·07
aβ, R III/IV = 1·85	0·95	9·55	1·68	−1·62
Low R III/IV = 1·68	0·48	1·87	0·48	−0·22
"−20", R III/IV = 2·01	0·05	4·30	0·14	−0·02

Pure porphyrin *a* yields, by definition, 100 p.c. values for porphyrin *a*, and no proto- or cryptoporphyrin by any of methods (c), (d) or (e) (Table 4, line 5). Investigations of porphyrin *a* preparations of abnormally low R III/IV made it possible to place these preparations in two classes. In the one represented by lines 7 and 2 of the Table, the low R III/IV was due to the presence of small amounts of proto- and cryptoporphyrin; these were crude preparations from which the other porphyrins could be readily removed. In other preparations, however, particularly those obtained by the earlier methods, the mathematical analysis of the spectrophotometric results gave large positive values for "protoporphyrin" and negative values for "cryptoporphyrin" (lines 3 and 4 of Table 4). From such preparations little spectroscopically pure porphyrin *a* could be obtained. Such preparations were also obtained from tissues other than heart muscle (Lemberg, Stewart and Bloomfield, 1955).

The yield of cryptoporphyrin a *from heart muscle.*

In contrast to the methods of Negelein and of Falk and Rimington which give large amounts of cryptoporphyrin (Lemberg, Bloomfield, Caiger and Lockwood, 1955), the methods described by us in that paper yield very little cryptoporphyrin according to the spectrophotometric analysis. The amounts varied between 0·1 and 3·8 mg. with an average of 1·6 mg. per kg. of fresh heart muscle, i.e., only one-eighteenth of the amount of porphyrin *a*. This neglects the small amount of cryptohaemin which may be removed in the crystals of protohaemin in this procedure (Lemberg, Bloomfield, Caiger and Lockwood, 1955, p. 439). It may be concluded from these experiments that cryptoporphyrin is an artifact, and from those described in the same paper on p. 442 and from the differences between cryptoporphyrin *a* and cryptoporphyrin *p* formed from haemoglobin (Lemberg, 1953; Parker, unpublished), that it is formed from porphyrin *a*.

SUMMARY.

Using the specific extinction values of porphyrin *a*, protoporphyrin and cryptoporphyrin *a* at four chosen wavelengths, spectrophotometric methods of analysis are worked out which allow the estimation of these porphyrins in mixtures of two or three of them.

The yields of cryptoporphyrin *a* from heart muscle, determined by one of these methods, varies with the procedure. With the methods developed by Lemberg, Bloomfield, Caiger and Lockwood (1955), it is little more than one-twentieth that of porphyrin *a*. Cryptoporphyrin *a* is probably an artifact derived from porphyrin *a*.

REFERENCES.

Granick, S. (1952): Fed. Proc., 11, p. 221.
Lemberg, R. (1953): Nature, 172, p. 619.
Lemberg, R., Bloomfield, B., Caiger, P. and Lockwood, W. H. (1955): Austral. J. exp. Biol., 33, p. 435.
Lemberg, R. and Stewart, M. (1955): *Ibid.*, 33, p. 451.
Lemberg, R., Stewart, M. and Bloomfield, B. (1955): *Ibid.*, 33, p. 491.
Stern, A. and Wenderlein, H. (1934): Z. physikal. Chem. A 170, p. 345.

PORPHYRINS WITH FORMYL GROUPS VIII

PRELIMINARY STUDIES OF THE PORPHYRIN *a* ISOLATED FROM MAMMALIAN TISSUES AND MICRO-ORGANISMS

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In a preceding paper (Lemberg, Bloomfield, Caiger and Lockwood, 1955, paper V of this series), methods have been described for the determination of the total haem *a* content of heart muscle in the form of porphyrin *a*.

The following paper describes some preliminary experiments carried out with other bovine tissues (skeletal muscle, kidney, liver, and udder) as well as with yeast and *Escherichia coli*.

EXPERIMENTAL.

Porphyrin *a* from heart muscle has been obtained in high yield in spectroscopically almost pure form by the methods described in the above mentioned paper. Success in the attempts to extract and measure haem *a* as porphyrin *a* from other bovine tissues and from micro-organisms was variable. Judged by the standards of purity adopted in the measurement of heart muscle porphyrin *a*, the isolated porphyrin *a* was less pure, and in some instances differed greatly from porphyrin *a*, particularly in the ratio of the extinctions of band III (558 mμ) and band IV (517 mμ) (R III/IV). This was usually far lower than in pure porphyrin *a* (2·04), or even the crude heart extracts (above 1·8). Pure porphyrin *a* could be isolated either only in small yield or not at all.

In order to investigate whether this was due to less complete separation of porphyrin *a* from other porphyrins, such as protoporphyrin or cryptoporphyrins, or to the presence of a porphyrin of type *a* different in structure and properties from that of heart muscle, two different methods were applied. Firstly there was used fractional extraction of the porphyrins from ether by hydrochloric acid of different concentrations and with phosphate buffer of pH 6·5, as described by Lemberg and Stewart (1955), for the separation of porphyrins a_α and $a\beta$ and the removal of some impurities, in paper VI of this series; secondly, the spectrophoto-

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metric analysis for mixtures of porphyrin *a*, cryptoporphyrin *a* and protoporphyrin developed by Lemberg and Parker (1955), in paper VII of this series. While this method indicated the presence of much protoporphyrin and cryptoporphyrin in such preparations of low R III/IV, and while in some instances, noticeably in the kidney, protoporphyrin could actually be demonstrated; in most other instances no independent evidence for the presence of proto- or cryptoporphyrin could be obtained. Furthermore, the spectrophotometric analysis using two different sets of extinctions (580, 555 and 513 mμ in one, and 555, 538, 513 mμ in the other) gave discordant results, whereas the results for mixtures of these porphyrins in heart muscle extracts were usually found to agree closely (Lemberg and Parker, 1955).

Skeletal muscle. 400 gm. of fresh veal steak (120 gm. dry weight) were treated according to method B_3 of paper V. After extraction of the porphyrin *a* with 20 p.c. HCl and driving back into ether by neutralization, ϵ_{555} mμ corresponds to a content of 12·8 mg. porphyrin *a* per kg. of dry weight calculated by the use of $\epsilon_{sp}^{558} = 20$ (see papers V and VI of this series). R III/IV=1·83. Fractional extraction from ether with phosphate buffer and hydrochloric acid yielded a "–20" fraction[1] of pure porphyrin *aa* (R III/IV=2·06). This formed, however, only 11 p.c. of the total, while in the other fractions R III/IV was about 1·8. Re-extraction of the ether solutions with 8 p.c. HCl failed to provide evidence for the presence of protoporphyrin or cryptoporphyrin *a*.

Kidney. 350 gm. of fresh ox kidney (63 gm. dry weight) were treated by method B_3 of paper V up to the stage of extraction of the other porphyrins with 8 p.c. HCl. At this stage ("crude porphyrin *a* fraction") total extinction (D_{555} mμ×ml./cm.) was 54, which would correspond to a yield of 43 mg. per kg. of dry weight. R III/IV was, however, only 1·36. Method B_3 was completed by extraction with 20 p.c. HCl and driving back into ether. At this stage the yield was only 12·7 mg. (per kg. dry weight), and R III/IV=1·40. If this ether solution was now once more extracted with 4 p.c. HCl, some protoporphyrin was extracted. The spectrum in ether showed that this fraction consisted mainly of protoporphyrin, accompanied by a trace of porphyrin *a*. The low R III/IV in this instance was therefore partly due to the difficulty of removing protoporphyrin in the first extractions with 4 p.c. and 8 p.c. HCl. Probably, this had been present in the form of an ester, perhaps of a higher fatty alcohol, which had been saponified by the treatment with 20 p.c. HCl.

Even after the treatment with 20 p.c. HCl, however, R III/IV in the "–15," "–20" and "+10" fractions remained low, varying between 1·60 and 1·84.

Liver. 370 gm. fresh ox liver (dry weight 130 gm.) were treated as before by method B_3. The yield calculated from D_{555} mμ of the crude porphyrin *a* fraction corresponded to 8·3 mg. per kg. dry weight, but R III/IV was only 1·0. 25 p.c. HCl extracted only an amount corresponding to 3·5 mg./kg. of R III/IV 1·34. No evidence was discovered for the presence of a protoporphyrin ester similar to that found in the kidney. The phosphate and hydrochloric acid fractionation gave porphyrin *a* of R III/IV 1·68 from the "+" and of R III/IV 1·55–1·72 in the "–10," "–15" and "–20" fractions, in a yield of about 50 p.c. of the total. A compound of low R III/IV was removed in the form of a precipitate at the interface of ether and 10–15 p.c. hydrochloric acid.

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[1]These symbols have been explained in the preceding paper of Lemberg and Stewart (1955).

In another experiment 720 gm. liver (210 gm. dry weight) were extracted with the addition of 50 mg. of α-tocopherol. In this experiment the freezing out of fat at −15° C. after raising the water content to 30 p.c. had been accidentally omitted. The yield of porphyrin *a* was small, and the 8 p.c. HCl fraction contained a considerable amount of cryptoporphyrin *a*. The analysis of Lemberg and Parker (1955) for all fractions indicated the total yield of 2 mg. porphyrin *a* per kg.

In two fractionations with phosphate buffer and hydrochloric acid, a "+5" fraction of R III/IV 2·01 (pure porphyrin *aβ*) was obtained, but in the "−15" fractions R III/IV was only 1·67. The "+10" and "−10" of the first fractionation had a low R III/IV of 1·0 and 1·26 respectively.

Cow udder. 520 gm. (130 gm. dry weight) were used. In this experiment the haematins had been precipitated from the acetone-HCl by neutralization with ammonia to a pH of 6·9. In the crude porphyrin *a* fraction, $D_{558\ m\mu}$ corresponded to a yield of 2·2 mg. per kg. of dry weight. R III/IV was 1·35. Analysis by the method of Lemberg and Parker (1955), using ϵ_{555}, ϵ_{538} and ϵ_{513} mμ, gave 2·8 mg. porphyrin *a*, 2·2 mg. cryptoporphyrin *a* and 12 mg. protoporphyrin per kg. The analysis indicated the presence of 30 p.c. cryptoporphyrin *a* and 18 p.c. of protoporphyrin in the "crude porphyrin *a*" fraction. An analysis, using the wavelength 580 mμ instead of 538 mμ did not give the same results, indicating that the fraction did not consist of a mixture of porphyrin *a* with cryptoporphyrin *a* and protoporphyrin, but contained a porphyrin different from porphyrin *a*. Nor could protoporphyrin or cryptoporphyrin be extracted by 8 p.c. HCl from the ether solution.

In a second experiment with 450 gm. cow udder (dry weight 115 gm.), R III/IV of the "crude porphyrin *a*" fraction was only 0·89, and was not increased greatly (to 0·93) by extraction with 25 p.c. HCl. Assuming $D_{558\ m\mu}$ to be due to porphyrin *a* alone, the yield would correspond to 2·3 mg. per kg. dry weight. The spectrophotometric analysis, according to Lemberg and Parker, indicated that this porphyrin consisted of about equal parts of cryptoporphyrin *a* with protoporphyrin, and contained only 10 p.c. of porphyrin *a*. This roughly agrees with the ratio $\epsilon_{580\ m\mu}/\epsilon_{513\ m\mu}$ of 0·67, which is that of protoporphyrin and cryptoporphyrin, while this ratio for porphyrin *a* is 1·19. Band IV is found at 510 mμ, in between the maxima of 513 mμ for cryptoporphyrin *a* and 503 mμ for protoporphyrin, while that of porphyrin *a* is 516–7 mμ. However, the absorption bands I and III occupied the positions they have in porphyrin *a* (646 and 558 mμ respectively), whereas they are found at 642 and 554 mμ in cryptoporphyrin *a* and 631 and 538 mμ in protoporphyrin. The latter two protoporphyrin bands were entirely missing. This porphyrin cannot, therefore, be a mixture of porphyrin *a*, cryptoporphyrin *a* and protoporphyrin. Either the porphyrin *a* has been altered during the preparation, or cow udder haemins *a* contain a prosthetic group different from those of heart muscle.

Yeast. 400 gm. compressed bakers' yeast (Mauri Bros. & Thomson Ltd.) were extracted with 80 p.c. acetone-20 p.c. water containing 0·7 p.c. hydrochloric acid without pre-extraction with 80 p.c. neutral acetone. The analysis of Lemberg and Parker indicated the presence of 1.35 gm. protoporphyrin, 0·29 mg. cryptoporphyrin, and only 0·05 mg. porphyrin *a* in 1 kg. of compressed yeast. Yeast autolysed with toluene before extraction of the haemins did not give higher yields (0·5 mg. protoporphyrin, 0·15 mg. cryptoporphyrin and 0·09 mg. porphyrin *a*). The crude porphyrin *a* fraction showed the following absorption spectrum: I a, 645; b, 629; II, 581; III, 556; IV, 505 mμ, with the minimum between band IV and the Soret band little marked. The band I a, band II and band III are those of porphyrin *a*, while band I b and band IV are close to those of protoporphyrin, but the band III of protoporphyrin at 538 mμ is missing.

Escherichia coli. In this organism the presence of chlorin derived from cytochrome a_2 produces an additional complication. 500 gm. (wet weight) of the centrifuged organisms

were used. From the ether solution of the porphyrins 4 p.c. HCl extracted protoporphyrin, and a trace of cryptoporphyrin *a*, indicated by a faint band at 555 mμ, after returning the porphyrin into ether. The first extract with 8 p.c. HCl contained predominantly chlorin (absorption band in ether 653 mμ); later extracts contained more cryptoporphyrin. 20 p.c. HCl yielded a mixture with absorption bands in ether at 652, 628, 576, 554, 532 and 500 mμ, consisting probably of a protoporphyrin-like porphyrin, chlorin and a small amount of porphyrin *a*.

DISCUSSION.

The results show that the porphyrin *a* isolated from tissues other than muscle differs from that obtained from heart by having a low R III/IV. The R III/IV of above 2·0 is not reached except for a fraction of the porphyrin from skeletal muscle and very small fractions of the porphyrin from liver. To some extent this may be due to difficulties of separating the relatively small amounts of porphyrin *a* quantitatively from other porphyrins. From our data (Lemberg and Parker, 1955) it can be shown that the value of $\epsilon_{555\ m\mu}/\epsilon_{513\ m\mu}$, which is close to R III/IV ($\epsilon_{558}/\epsilon_{517}$) in porphyrin *a*, is lowered from 2·01 to 1·86 by the admixture of as little as 5 p.c. protoporphyrin or 10 p.c. cryptoporphyrin *a*, to 1·74 by 10 p.c. protoporphyrin, and to 1·61 by 15 p.c. protoporphyrin, or 10 p.c. protoporphyrin+10 p.c. cryptoporphyrin. In the kidney we have, indeed, found evidence for the presence of a protoporphyrin derivative, which gave protoporphyrin on saponification with strong hydrochloric acid. In yeast and *E. coli* the spectrum of the crude porphyrin *a* contains bands resembling those of protoporphyrin, but the low basicity of the porphyrin and the lack of band III at 538 mμ show that protoporphyrin is not present. Similar observations have been made with Corynebacterium diphtheriae by Rawlinson and Hale (1949), except that they found a III b. band at 541 mμ.

Such admixture of other porphyrins does not explain the low R III/IV values entirely, however. Either the porphyrin *a* of the cytochromes of other tissues differs from those of heart and muscle, or alterations of porphyrin *a* occur during the isolation from other tissue which we have been able to avoid with heart muscle. The latter appears at present to be the more likely explanation, since porphyrin *a* of low R III/IV has also been found in heart muscle prepared by the earlier procedures, or by removal of iron with large excess of ferrous acetate.

While this alteration of, or difference from, porphyrin *a* introduces some uncertainties in the quantitative results of the analysis of the mammalian tissues, the errors are probably not large with the exception of the values for kidney.

In Table 1 are summarized the porphyrin *a* contents of the analysed bovine tissues, as found in this and the preceding study (Lemberg, Bloomfield, Caiger and Lockwood, 1955) and their cytochrome *c* contents according to Junowicz-Kocholaty and Hogness (1939). The values above are those determined after

extraction of porphyrin *a* with 20–25 p.c. HCl from ether. These are probably too low. The values obtained before HCl extractions are 1·2–3·3×higher, but are not so reliable for organs other than heart. QO_2 values of bovine tissues have not been studied as far as we know. Hence, QO_2 values are given for rat tissues, together with their cytochrome *c* contents according to Drabkin (1949), with the addition of a value for working sheep heart *in situ* which can be derived from measurements of Edwards *et al.* (1954). If one can draw conclusions from the rather unsatisfactory use of approximate QO_2 values for the tissue of another animal, it would appear that in general the cytochrome *c* content parallels QO_2, and that the yield of porphyrin *a* parallels the cytochrome *c* content, except that the porphyrin *a* content of cow udder appears to be abnormally low.

TABLE 1.

Porphyrin *a*, cytochrome *c* and QO_2 of mammalian tissues.

Tissue	Bovine			Rat	
	Porphyrin *a*		Cytochrome *c*	Cytochrome *c*	QO_2
	μ moles/kg. before	dry wt.[1] after HCl extraction	μ moles/kg. dry wt.[2]	μ moles/kg. dry wt.[3]	μl. O_2/mg. dry/hr.
Heart	90	74	53	149	>20 (48)[4]
Skeletal muscle	—	15	16	29	6
Kidney	50?	15	12	110	20
Liver	10?	4	4	47	<10
Mammary gland	abt. 3	—	—	—	9[5]

[1] From $\epsilon_{558m\mu}$, assuming $\epsilon^{558\ m\mu}_{mM} = 17$.
[2] Calculated from values (mg./kg. fresh) of Junowicz-Kocholaty and Hogness (1939).
[3] Calculated from values (mg./kg. dry) of Drabkin (1949).
[4] Calculated from the results of Edwards *et al.* (1954) for working sheep heart *in situ*.
[5] Folley and French (1949). In cow's mammary gland 3·5.

The cytochrome *c* content of ox liver and kidney is remarkably low when compared with that of rat liver and kidney. Studies on the porphyrin *a* content of rat tissues are being carried out.

In yeast, the method evidently fails to remove large parts of porphyrin *a* from the cytochromes. According to the cytochrome a_3 content of bakers' yeast, determined spectrophotometrically by Smith (1954), the yield of porphyrin *a* from bakers' yeast should be 25 mg. per kg. of dry weight, whereas only 0·3 mg. of still impure porphyrin *a* could be isolated by us. It is possible that our yeast contained a smaller concentration of cytochromes, but the cytochrome spectrum was marked and the cytochrome *a* band clearly visible.

SUMMARY.

The method used for the isolation and estimation of porphyrin *a* of heart muscle has been applied to bovine tissues as well as to bakers' yeast and *E. coli*.

Only from skeletal muscle was nearly pure porphyrin *a* obtained in good yield; further fractionation yielded pure porphyrin *a* in small yield. Pure porphyrin *a* was also obtained from liver, in very small yield. The porphyrin *a* from kidney, liver and cow udder differed from that of heart in its absorption spectrum (low ratios of bands III to band IV), probably because it is altered during the extraction. Only a very small fraction of porphyrin *a*, highly impure, was obtained from yeast and *E. coli*.

Evidence has been found in kidney for the presence of a protoporphyrin ester which is not extracted from ether by 8 p.c. HCl, but which can be converted to protoporphyrin by strong HCl. Protoporphyrin-like compounds also accompany porphyrin *a* in the extracts from the micro-organism, but these were not converted to protoporphyrin by saponification with acid.

REFERENCES.

Drabkin, D. L. (1949): "Aspects of the Oxygenation and Oxidation Functions" in "Haemoglobin," ed. by F. W. Roughton and J. C. Kendrew, Butterworths Scientific Publications, London, p. 35.

Edwards, W. S., Tulny, S., Reber, W. E., Siegel, A. and Bing, R. J. (1954): Ann. Surg., 139, p. 275.

Folley, S. J. and Francy, T. H. (1949): Biochem. J., 45, p. 117.

Junowicz-Kocholaty, R. and Hogness, T. R. (1939): J. biol. Chem., 129, p. 569.

Lemberg, R., Bloomfield, B., Caiger, P. and Lockwood, W. H. (1955): Aust. J. exp. Biol., 33, p. 435.

Lemberg, R. and Parker, J. (1955): *Ibid.*, 33, p. 483.

Lemberg, R. and Stewart, M. (1955): *Ibid.*, 33, p. 451.

Rawlinson, W. A. and Hale, J. H. (1949): Biochem. J., 45, p. 247.

Smith, L. (1954): Arch. Biochem. Biophys., 50, p. 285.

THE ACTION OF YOHIMBINE ON NERVE AND MUSCLE OF AMPHIBIA

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In a previous paper (MacCallum, McCallum and Shaw, 1949) it was shown that an essential feature of the yohimbine nerve block was a marked increase in the absolute refractory period or, in other words, an increase in the recovery time. The yohimbinized nerve is capable of transmitting single impulses, but the spike height progressively declines to zero with repetitive stimulation. Recovery, however, will take place within a second of cessation of stimulation.

Associated with this repetitive decline, there invariably occurs a lowering of conduction velocity. In the initial stages of its action, yohimbine raises the threshold to stimulation, leaving the conductivity unaffected. Later, however, the nerve is completely blocked, and prolonged washing in Ringer's solution is required for its recovery.

Graham (1935) has shown that yohimbine augments the positive after-potential; while Shanes (1951) records that yohimbine depresses spontaneous activity, and that, like procaine, it slightly increases the resting potential of frog sciatic nerve, delays anoxic depolarization, and decreases hyperpolarization in carbogen. Simon (1955) has shown that the resting ionic pattern of muscle and nerve is unaffected by the alkaloid, and that the active movements of Na and K ions against a gradient are not hindered.

The work to be described in the present paper falls into two parts. Firstly, the effect of yohimbine on certain biophysical characteristics of nerve and muscle is described; and secondly, the effect on yohimbine action of a variation in the external ionic environment is noted.

Yohimbine has little effect on the biophysical properties of nerve or muscle; notably the membrane potential of muscle is not altered. The action of the alkaloid is altered by variations in external ionic pattern, particularly by diminution in the value of the external sodium.

MATERIALS AND METHODS.

The tissues of the toad, *Bufo marinus*, were the usual source of biological material. The layout of the nerve preparation followed the plan described in the previous paper (MacCallum,

[1]This work was carried out with the aid of a grant from the National Health and Medical Research Council of Australia.

McCallum and Shaw, 1949). The yohimbine was used as 1:1000 solution of the hydrochloride in Ringer solution and was applied to a 1 cm. length of nerve between the stimulating and pick-up electrodes, which were usually about 5 cm. apart. The stimulus was adjusted for maximal A fibre response.

Apparatus was developed for the measurement of the resting potential (R.P.) and action potential (A.P.) of toad sartorius muscle with intracellular electrodes (3 M KCl, micro capillary type). The recording equipment consisted of an input head of a similar type to that described by Nastuk and Hodgkin (1950), but with a second cathode follower as a buffer stage.

Two D.C. amplifiers were used. One drove an Evershed and Vignoles type chart recorder and was used to measure the resting potential (R.P.). The output of the second was connected to the CRO. The working range of the apparatus was checked regularly for linearity, and routine calibration was carried out with a standard cell and precision resistors.

A square wave voltage generator was used for stimulation—its output being isolated from the bath by a special transformer. It also provided a square pulse which could be fed through the indifferent electrode and used to check the input rise time of the apparatus. This varied from 75 to 150 μ seconds, depending on the resistance of the tip of the micro-electrodes. The stimulus was applied by means of external electrodes.

Microelectrodes were pulled by hand in a gas and oxygen flame. They were placed in cold filtered KCl and the tips filled by capillary action. The shanks were filled by means of a thin Pasteur pipette and the remaining air bubble removed with a fine glass plunger. The tips could not be resolved with critical white light and their resistances varied from 5 to 15 M ohm. Ag/AgCl electrodes were used at both liquid:metal interfaces. A pair of movable platinum electrodes was used for stimulating.

The muscle rested on the bottom of a shallow perspex bath and in most of the experiments it was not secured in any way. All procedures were carried out under microscopic control.

Criteria of successful penetration came from microscopic observation and from electrical readings: the most useful being the rapid rate of change of potential as the electrode entered the fibre. Once successfully impaled, small movements of the electrode did not cause any change in R.P. If the fibre contracted during penetration it was assumed that some damage had occurred and the reading was disregarded. Normal fibres could always be stimulated and gave consistent action potentials (A.P.).

It was more difficult to decide whether a penetration was satisfactory when working with yohimbinized muscles, since the A.P. was no longer normal.

The nature of the connective tissue which invests bundles of fibres throughout toad muscle varied considerably between different specimens (generally becoming increasingly tough as the animals were kept in captivity). It was occasionally difficult to decide whether a low R.P. resulted from blocking of the tip by a thin sheet of connective tissue, from an injured fibre or from drug action.

Solutions.

(a) "Phosphate" Ringer		(b) Bicarbonate Ringer
NaCl	128 m.eq./l.	128 m.eq./l.
KCl	2·7	2·7
$CaCl_2$	1·08	1·08
$NaHCO_3$	—	9
Phosphate	10	0·3
Dextrose	22	22
Oxygen	100 p.c.	5 p.c. CO_2 in O_2

RESULTS.

Repetitive decline—nerve. A standard repetition rate of 30 impulses per second was employed throughout. Under these circumstances, the time of yohimbinization required for full repetitive block was between 60 and 70 minutes at 15° C.

At this point, when a full decay to zero was first observed, single spikes remained of normal maximal height, and the preparation showed, within 0·5 seconds, recovery following repetitive block.

Fig. 1a illustrates this phenomenon, and the velocity change shown represents a decrease from 39 to 22 metres per second.

A nerve soaked in weak yohimbine (1:20,000) overnight still transmitted one or two impulses before a block occurred. A period of about 3–5 minutes was necessary before the nerve was able to conduct another impulse.

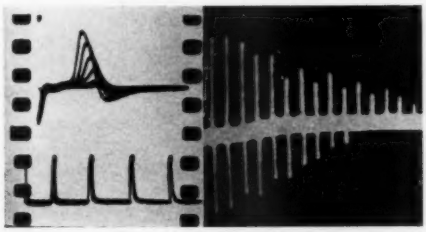

Fig. 1a. Yohimbine 1:1000. Complete fatigue at 4 shocks per second. Calibration (below): Vertical, 10 m. volts; horizontal, each space 2 m. secs.

Fig. 1b. Same as Fig. 1a with reduced sweep speed.

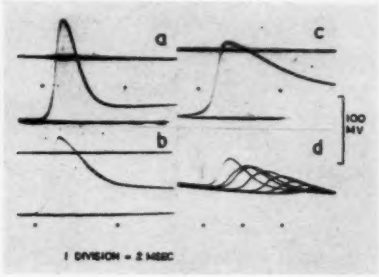

Fig. 2. The effect of yohimbine on the A.P. recorded intracellularly.
(a) Normal A.P.
(b) A.P. from "normal" fibre after prolonged repetitive stimulation (10 mins. at 100/sec.).
(c) After 1 hour in yohimbine 1:50,000 —3rd stimulus.
(d) Repetitive stimulation (stimulus rate ¼/sec.).

When recorded with slow sweep, the record appears as in Fig. 1b. The general decline in spike height is seen to have minor oscillatory phenomena associated with it.

These are records of A-fibre transmission. On the other hand, B-fibres were found to be much more sensitive to yohimbinization, being completely blocked after 20 minutes' treatment. This blocking phenomenon displayed the same general features as previously observed with A-fibres.

The sensitivity of C-fibres to yohimbinization was found to be of the same order as that of A-fibres, both groups requiring 70 minutes' treatment with the drug for complete repetitive block to occur.

Temperature coefficient—nerve. Using the time taken for a fresh whole nerve first to show complete block under repetitive stimulation of 20 impulses per second, the temperature coefficient for yohimbinization between 15° C. and 25° C. was found to be 2·3. This suggests that the yohimbine combines with some chemical receptor.

Polarization—nerve. Polarizing electrodes were set up, one on the nerve in the drug bath and the other distal to the stimulating electrodes. The e.m.f. used was 1·5 volts from a dry cell.

The procedure was to produce repetitive block in the yohimbinized nerve, and then, maintaining the stimulation, to key in the polarizing voltage.

Under these circumstances, yohimbine inhibition was found to be temporarily relieved by anodal polarization, but not by cathodal.

The anodal relief was not an instantaneous effect, the spikes appearing after about 3 seconds' polarization.

Spread of impulse on the block—nerve. An experiment was carried out which was identical in principle with that described by Hodgkin (1937). This experiment elucidated the effect of a blocked impulse on the region of the nerve immediately distal to the block. The electrotonic spread beyond the block was measured. This spread extended for a distance of 7 mm.

By means of a dual pulse stimulator, the lowered threshold beyond the drug-treated region of the nerve was shown to correspond with the above region of extrinsic potential.

Resting potential—muscle. A long series of measurements (over 500) of normal R.P. in phosphate Ringer gave an average value of 90·4 MV with a S.E.±0·456. Bicarbonate Ringer gave an average of 93±0·35 MV. The muscle was soaked in concentrations of yohimbine from 1:1000 to 1:5000 in phosphate or bicarbonate Ringer.

In some cases readings were begun immediately the muscle was placed in the yohimbine solution. In all cases the R.P. was determined for at least two hours after the addition of the drug. At a concentration of 1:1000 a difference in appearance of the muscle was detected in several experiments after 1 to 2 hours' soaking in yohimbine. The surface fibres became "cloudy." They seemed tough and were difficult to penetrate. Macroscopically yohimbine at a concentration of 1:1000 was found, after a period of 1 to 2 hours, to produce bunching and rigor in the muscle.

A decrease in R.P. of up to 10 MV was observed with a concentration of 1:1000, but this may have been due to difficulty of penetration. The fall in R.P. was less marked in phosphate Ringer—in one experiment no change could be observed after 2 hours' soaking in yohimbine.

In more dilute solutions (1:5000) little change in the R.P. could be detected. On averaging values obtained over a number (200) of experiments, no significant increase could be found.

In none of these cases were we able to demonstrate a hyper-polarization of the order of magnitude reported by Shanes (1951). Our results suggest that yohimbine should not be classified as a hyperpolarizing drug.

Action potential—muscle. All the properties of yohimbine block in nerve described by Shaw *et al.* (1949) were seen on yohimbinized toad muscle. Intracellular recordings, however, revealed some further details. The fibres near the surface of the muscle were very sensitive to the drug, responding to doses as low as $1:10^5$. Block occurred far more rapidly in muscle, due, probably, to the lack of a diffusion barrier such as the sheath which surrounds toad nerve.

On investigating the nature of the block a constant procedure was used. After control readings had been taken, the muscle was placed in the drug solution for various times. It was then carefully removed and placed in the testing bath and the stimulating electrodes placed in position. A limited number of single stimuli (supra maximal) were applied directly to the muscle and the A.P. recorded in each case. The muscle was then stimulated repetitively first with shocks at frequencies of about $\frac{1}{4}$/sec. and in some cases (low yohimbine concentrations) up to 20/sec. until block was complete. Single shocks to yohimbinized muscle resulted in a change in the nature of the A.P. A typical example is shown in Fig. 2.

After one hour in 1:50,000 yohimbine the spike height was reduced to 70–80 p.c. of normal. There was almost no alteration in the rate of rise of potential but a very marked increase in the rate of fall. Higher concentrations (up to 1:2000) reduced the height still further (to about 60 p.c.), block was usually complete after the first two or three single stimuli and recovery in the yohimbine solution took several minutes. As in yohimbinized nerve, block was accompanied by a decrease in the velocity of propagation and a marked increase in the width of the spike (see Fig. 2).

In normal Ringer surface fibres which were blocked after 1 to 2 hours in $1:10^5$ yohimbine, returned to their original form in $\frac{1}{2}$ to 1 hour.

Effects of Ringer composition.

Sodium—nerve. Replacement of the sodium chloride component of the Ringer solution by 2·5 p.c. choline chloride solution produced a solution capable of blocking nerve transmission in 7 to 8 hours. Repetitive stimulation greatly enhanced the effect, and the block was similar in every way to that produced by yohimbinization.

Fig. 3 illustrates how this effect was obtained using a double-beam oscillograph, and pick-up from both peroneal and sciatic branches of the sciatic nerve.

It is clear from this experiment that deterioration of the excised nerve is not the causal factor in the block.

It was then shown that pre-treatment of the nerve in sodium-free solutions,

which alone had no detectable effect on conduction, greatly enhanced the blocking action of yohimbine.

The sodium block, resembling yohimbine block in the repetitive decline of A.P., velocity shift, relief by anodal polarization, rapid recovery for single spikes, and lack of effect on membrane potential of resting nerve, nevertheless showed two distinguishing features.

A nerve once blocked by sodium deficiency and later restored by addition of sodium, displayed no signs of increased sensitivity either to sodium deficiency

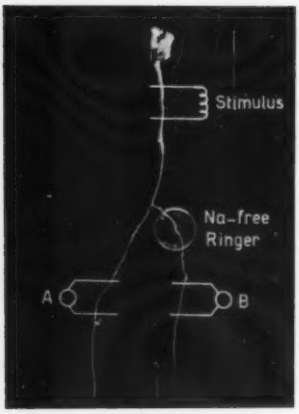

Fig. 3. Effect of sodium-free solution on nerve. The mixed sciatic was stimulated. The impulse picked up at A (peroneal branch) was not affected during the time of the experiment (6 hours). There was an effect resembling yohimbine action recorded at B (sciatic branch).

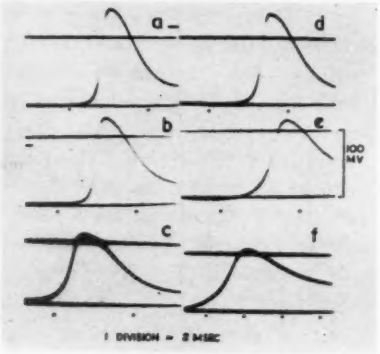

Fig. 4. The effect of low sodium on the A.P. (muscle).
(a) 128 m.eq./l. of Na — immediate effect.
(b) 85 m.eq./l. of Na — immediate effect.
(c) 34 m.eq./l. of Na — immediate effect.
(d) 85 m.eq./l. of Na — after 2 hours' soaking.
(e) 34 m.eq./l. of Na — after 2 hours' soaking.
(f) 17 m.eq./l. of Na — after 1 hour's soaking.

or to yohimbinization. A yohimbinized nerve, however, after full restoration, subsequently showed an increased sensitivity to both these agents.

Secondly, prolonged washing was required to achieve recovery in yohimbinized nerve, whereas addition of sodium produced a rapid return to normal of sodium-deficient nerves.

This difference between sodium deprivation and yohimbine block may not be so marked as it first appears. If the yohimbine is reproducing a sodium deficiency by interference with an enzyme system, then one would not expect a rapid reversal on removal of the drug.

The extreme length of time required for sodium block in whole nerve was decreased to the order of 30–40 minutes by incising the epineurium of the portion of the nerve in contact with the test solution. Similar treatment of the nerve reduced the time of yohimbine block to one-third.

Increasing the sodium concentration in the Ringer's solution up to 200 mM/litre had the effect of slightly increasing the height of the action potential, and delaying somewhat the action of yohimbine. The drug produced the full repetitive decline in about 80–90 minutes in the presence of this sodium excess.

Sodium—muscle. Intracellular recordings showed several differences between the A.P. recorded with repetitive stimulation in Na-deficient solutions and that recorded in yohimbine solutions. The A.P. to single stimuli after soaking for 1–2 hours in solution low or deficient in Na showed a marked decrease in rise time. Block occurred at relatively low rates of stimulation (10/sec. after 2 hours in 34 m.eq. Na/litre). The most marked feature of these A.P. was an ever greater decrease in rise time than that seen on single stimulation (Fig. 4).

A full exposition of the effect of Na lack on the A.P. will be given in a later paper (Holman *et al.*, 1955).

A.P. more closely resembling those seen in yohimbinized fibres were observed after a muscle had been fatigued by prolonged stimulation (rate 100/sec.) in normal Ringer's solution. These are shown in Fig. 2b. The time of stimulation required before such "fatigued" A.P. could be observed, varied considerably among different specimens (5 to 20 mins.). The time taken for the A.P. of such fibres return to the shape of a normal A.P. also varied, but was generally brief (1 to 5 mins.).

Potassium—nerve. Nerves soaked for 4 hours in potassium-free solutions showed a slight increase in spike height. Potassium deficiency, however, decreased the mean time of yohimbine inhibition from 65 mins to 40 mins. Prior soaking in potassium-free solutions for 1 hour slightly increased this effect, so that block occurred in 25 mins.

A three-fold increase in the potassium concentration of Ringer's solution (i.e., up to 9·6 mE.) produced a decrease in spike height after about 2½ hours. The spike fell to 65 p.c. of its normal height and remained constant thereafter. Progressive increase in potassium concentration produced proportionately more rapid yohimbine block. 9·6 mE. solutions which are not depolarizing, decreased the yohimbine time from 65 to 30 mins.

Calcium—nerve. Calcium lack reduces the time of yohimbine inhibition from 65 mins. to 45 mins. Pre-treatment with calcium-free solution did not enhance this effect.

Increase in calcium concentration up to 20 mE. (i.e., 15-fold) were required to cause conduction block. The rate of yohimbine block was only slightly increased by excess calcium.

In a few instances, calcium-blocked nerves showed a tendency to repetitive decline of the yohimbine type, but this was not a general phenomenon.

Magnesium—nerve. Lack of magnesium in the Ringer's solution had no effect on the height of the normal spike, nor upon the rate of yohimbine block.

A 10-fold increase in magnesium concentration, however (i.e., 12 mE.), while not affecting the spike alone, decreased the time of yohimbine block from 65 to 20 mins.

DISCUSSION.

The action of yohimbine on nerve is extremely interesting. A treated nerve is able to conduct impulses but requires a longer period for recovery between stimuli. A nerve treated for as long as 24 hours is quite able to conduct one or two impulses normally before block occurs and will then require a rest period of up to 5 minutes. It would seem that the resting nerve is unaffected by yohimbine and that only the changes taking place during activity (conduction) or at the end of activity are sensitive to yohimbine.

That yohimbine is without effect on resting cells is borne out by (a) an absolute conduction block is difficult to produce, (b) the resting Na and K contents of soaked muscle are unaltered (Simon, 1955), (c) the resting potential of single muscle fibres is unaffected, (d) oxygen consumption is only slightly reduced (unpublished observation).

That yohimbine affects active cells is shown by (a) conduction block on repetitive stimulation, (b) the action potential of single muscle fibres is changed.

Thus, in yohimbine, we have a drug which is capable of separating the changes which take place in resting tissue from those which take place when the tissue is active.

When one comes to discuss the mode of action of yohimbine on active cells the results are more equivocal. If one considers only the relationship between yohimbine and sodium on nerve one would be justified in saying that yohimbine interferes with Na metabolism in the active cell. That is to say, Na lack mimics yohimbine effect and Na excess mildly counteracts it.

However, the alteration of the other ions in the media does not yield a clear picture. Increasing or decreasing the amounts of potassium, calcium or magnesium either has no effect on the action of yohimbine or increases the rate of its action. Particularly puzzling is the fact that both K-free and K-rich Ringer's enhance the effect of yohimbine. Previous work (Shaw and Simon, 1955) suggests that the intracellular Na is unaltered in K-free media. Yet both treatments enhance the action of yohimbine. Likewise it has been shown (Shaw and Simon, unpublished) that a Ca-free Ringer does not alter the Na content of nerve to any great extent, yet such a solution enhances the action of yohimbine. The explanation could be advanced that alteration in ionic environment leads to an increased accessibility of yohimbine. In other words, marked alteration of the ionic environment may break down a diffusion barrier in the nerve so that it is more susceptible to the action of the drug.

The inference that yohimbine interferes with the Na metabolism but not with K exchanges is confirmed by the fact that this alkaloid does not affect the resting potential in muscle which, according to the modern concepts, is a "potassium potential," but it does alter the action potential which is a "sodium potential" (Eccles, 1954).

In a previous paper (Simon, 1955) it was shown that ionic shifts of Na and K taking place in excised resting muscle were unaffected by yohimbine. The mimicking of Na deficiency by yohimbine is not so pronounced with muscle as it is with nerve. Indeed, in the case of muscle, yohimbinization mirrors fatigue rather than Na deficiency. In both instances activity of the tissue is necessary before yohimbine action can take effect, and it has always appeared to us that yohimbine hinders some recovery process in active tissue. Such an hypothesis could be used to account for the resemblance between fatigued and yohimbinized muscle. As nerve is unfatiguable such a likeness could not be perceived in normal nerve, but certainly a yohimbinized nerve appears to be "fatigued."

In conclusion then, it would appear that yohimbine is a non-toxic, readily reversible agent for investigating the mechanism of changes associated with an active cell while at the same time leaving unaffected the background interchanges of the resting cell.

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REFERENCES.

Graham, H. (1935): Amer. J. Physiol., 111, p. 452.
Hodgkin, A. L. (1937): J. Physiol., 90, p. 183.
Holman, Mollie E., Shaw, F. H. and Simon, Shirley E. (1955): In press.
MacCallum, M., McCallum, I. and Shaw, F. M. (1949): Austral. J. exp. Biol., 17, p. 115.
Nastuk, W. and Hodgkin, A. L. (1950): J. cell. comp. Physiol., 35, p. 39.
Shanes, A. M. (1951): *Ibid.*, 38, p. 17.
Shaw, F. H. and Simon, Shirley E. (1955): Austral. J. exp. Biol., in press.
Simon, Shirley E. (1955): *Ibid.*, in press.